KARYOTYPING IN CHRONIC MYELOID LEUKEMIA

Dissertation submitted in partial fulfilment
of the requirements for the degree of

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MADRAS MEDICAL COLLEGE,
CHENNAI – 600 003.

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CHENNAI

APRIL 2015

CERTIFICATE

This is to certify that this Dissertation entitled **"KARYOTYPING IN CHRONIC MYELOID LEUKEMIA"** is the bonafide original work of **Dr.S.DEVIPANGAJ,** in partial fulfillment of the requirement for M.D., (Branch III) in Pathology examination of the Tamilnadu Dr.M.G.R Medical University to be held in April 2015.

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I **Dr.S.DEVIPANGAJ,** solemnly declare that the dissertation titled **"KARYOTYPING IN CHRONIC MYELOID LEUKEMIA"** is the bonafide work done by me at Institute of Pathology, Madras Medical College under the expert guidance and supervision of **Prof.Dr.GEETHA DEVADAS M.D.,D.C.P.,** Professor, Institute of Pathology, Madras Medical College with co-guidance of **Prof.Dr.USHA, M.D.,D.M.,** Professor and Head of the department of Hematology, Madras Medical College. The dissertation is submitted to the Tamilnadu Dr.M.G.R Medical University towards partial fulfillment of requirement for the award of M.D., Degree (Branch III) in Pathology.

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To
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Dear Dr. Devi Pangaj,

The Institutional Ethics Committee of Madras Medical College, reviewed and discussed your application for approval of the proposal entitled **"Karyotyping in Chronic Myeloid Leukemia"** No.11062014

The following members of Ethics Committee were present in the meeting held on 03.06.2014 conducted at Madras Medical College, Chennai-3.

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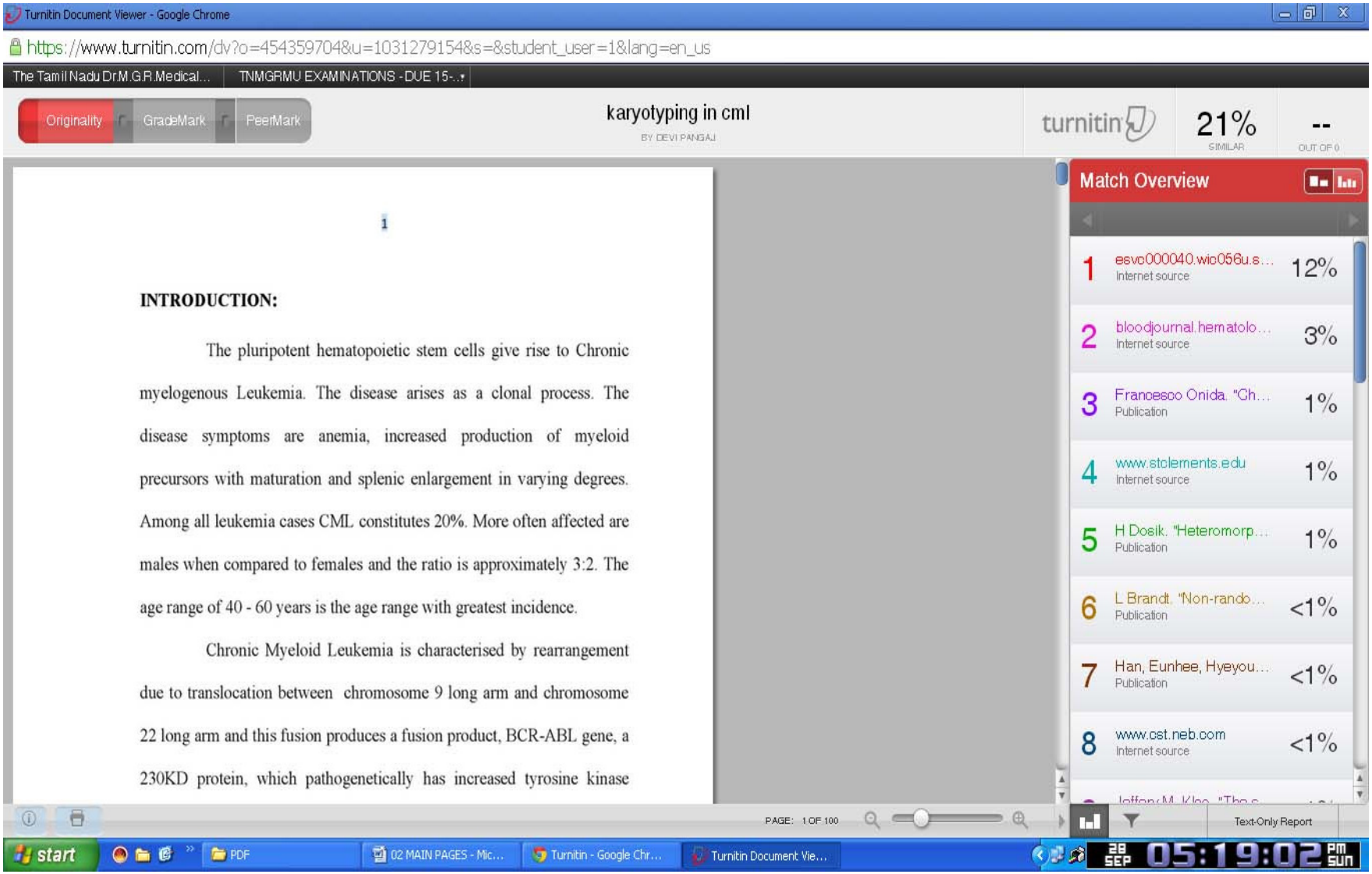
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The pluripotent hematopoietic stem cells give rise to Chronic myelogenous Leukemia. The disease arises as a clonal process. The disease symptoms are anemia, increased production of myeloid precursors with maturation and splenic enlargement in varying degrees. Among all leukemia cases CML constitutes 20%. More often affected are males when compared to females and the ratio is approximately 3:2. The age range of 40 - 60 years is the age range with greatest incidence.
Chronic Myeloid Leukemia is characterised by rearrangement due to translocation between chromosome 9 long arm and chromosome 22 long arm and this fusion produces a fusion product, BCR-ABL gene, a 230KD protein, which pathogenetically has increased tyrosine kinase
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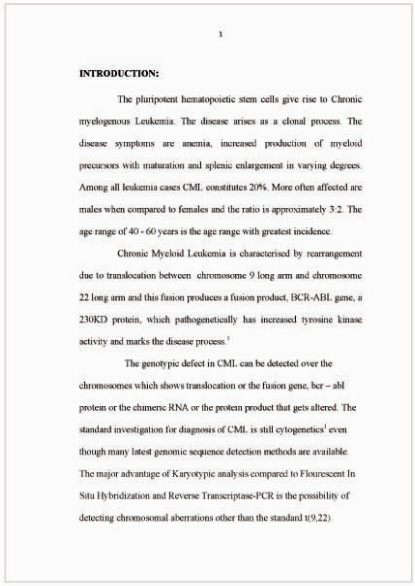

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Chronic Myeloid Leukemia is characterised by rearrangement due to translocation between chromosome 9 long arm and chromosome 22 long arm and this fusion produces a fusion product, BCR-ABL gene, a 230KD protein, which pathogenetically has increased tyrosine kinase activity and marks the disease process.[1]

The genotypic defect in CML can be detected over the chromosomes which shows translocation or the fusion gene, bcr – abl protein or the chimeric RNA or the protein product that gets altered. The standard investigation for diagnosis of CML is still cytogenetics[1] even though many latest genomic sequence detection methods are available. The major advantage of Karyotypic analysis compared to Flouresecent In Situ Hybridization and Reverse Transcriptase-PCR is the possibility of detecting chromosomal aberrations other than the standard t(9,22)

ABBREVIATIONS

CML	:	Chronic Myeloid Leukemia
ACA	:	Additional Chromosomal Abnormality
V, t	:	Variant translocation
+8	:	Trisomy 8
+19	:	Trisomy 9
Inv 12	:	Inversion 12
-Y	:	Deletion Y chromosome
DNA	:	Deoxy ribonucleic acid
IHC	:	Immunohistochemistry
PCR	:	Polymerase chain reaction
RT PCR	:	Reverse Transcriptase Polymerase Chain Reaction
ISCN	:	International System of Cytogenetic Nomenclature
FISH	:	Fluorescent in situ hybridization
cDNA	:	complementary deoxy ribonucleic acid
mRNA	:	Messenger ribonucleic acid
BCR	:	Breakpoint Cluster Region
ABL	:	Abelson murine leukemia virus
Ph	:	Philadelphia

CONTENTS

S. NO.	TITLE	PAGE NUMBER
1	INTRODUCTION	1
2	REVIEW OF LITERATURE	3
3	AIMS AND OBJECTIVES	66
4	MATERIALS AND METHODS	67
5	OBSERVATION AND RESULTS	80
6	DISCUSSION	95
7	SUMMARY	103
8	CONCLUSION	105
	BIBILIOGRAPHY ANNEXURES MASTER CHART	

KARYOTYPING IN CHRONIC MYELOID LEUKEMIA

ABSTRACT

The prognostic relevance of additional cytogenetic findings at diagnosis of Chronic Myeloid Leukemia (CML) is unclear. The impact of additional cytogenetic findings at diagnosis on time to progression free survival and clinical and laboratory features along with disease course is analysed in 30 cases of Chronic Myeloid Leukemia and compared with patients presenting with standard t(9,22)(q34,q11.2) only karyotype. 6 cases (20%) were found to have abnormal karyotype at diagnosis, of which 4(13.33%) were additional chromosomal abnormality(ACA), 1 patient (3.33%) had complex variant karyotype and 1 patient (3.33%) had –Y. On median observation for 15 months, the patients with ACA were found to have accelelaration of disease, abnormally high counts, renal compromise and unexplained thrombocytopenia and this indeed signifies the importance of cytogenetic diagnosis at initial diagnostic workup and the impact they have on progression of disease and prognosis.

INTRODUCTION

The pluripotent hematopoietic stem cells give rise to Chronic myelogenous Leukemia. The disease arises as a clonal process. The disease symptoms are anemia, increased production of myeloid precursors with maturation and splenic enlargement in varying degrees. Among all leukemia cases CML constitutes 20%. More often affected are males when compared to females and the ratio is approximately 3:2. The age range of 40 - 60 years is the age range with greatest incidence.

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Situ Hybridization and Reverse Transcriptase-PCR is the possibility of detecting chromosomal aberrations other than the standard t(9,22) Philadelphia such as major and minor route additional chromosomal abnormalities and variant translocations that may have impact on prognosis.[1]

Cytogenetic studies reveal the Philadelphia chromosome which corresponds to translocation between chromosome 9 and 22, is seen in 90-95% of CML, whereby chromosome 22 appears to be 'shortened'. Reciprocal translocation t(9;22)(q34.1;q11.2) whose product is standard Philadelphia constitutes more than 90% of the Ph translocation. Variant translocations is seen in remaining 5 -10% patients which may be complex type or simple type. High-resolution banding studies show cryptical involvement of translocation of 9q34 to 22q11 in simple and complex variant translocation. Additional chromosomal aberrations or ACA occurring with disease progression is estimated to be 75-80%. In few cases, ACA can occur even at the intial disease course itself and if at all they are diagnosed at the beginning, the worse course they impart could be missed and appropriate management measures could not be taken. The presence of ACA at disease onset signifies a poor prognosis.[3]

REVIEW OF LITERATURE

In the era of twentieth century, the classic myeloproliferative disorders (MPDs) like, primary myelofibrosis (PMF), chronic myelogenous leukemia (CML), essential thrombocythemia and polycythemia vera were described.[4,5,6,7,8,9] These four pathologic entities were classified by William Dameshek, along with others in 1951 as 'MPDs'.[10] The MPDs history even antedates Dameshek by a century or more and from his time many developments are made in discovering the disease process, including *BCR–ABL*[11,12,13,14] and *JAK2*V617F.[15,16,17,18,19]

Van Leeuwenhoek made the definition of the red blood cell and William Hewson (1739–1774) English physicians, the white blood cells and he is known as 'Father of Hematology'.[14,15]

In 1845, English pathologist John Hughes Bennett (1812–1875), reported the first case of Chronic Myeloid Leukemia titled “Case of hypertrophy of the spleen and liver in which death took place from suppuration of the blood”.[16]

It was Virchow in 1847 who named the new entity as 'Leukhemia', while Bennet said its 'Leucocythaemia' . Despite controversy, Virchow ceded priority to Bennett ultimately.

Difference between granulocytic and lymphatic variants of Leukemia were described by Virchow. In 1868, a German pathologist, Ernst Christian Neumann, putforth the theory of formation of blood cells from marrow of bone[24]. Leukemias were later classified into myeloid and lymphoid subtypes in 1880 by Paul Ehrlich who described the concept of common stem cell & it's progeny into distinct cell lineages.[25]

William Dameshek, a Russian by birth, along with Henry M Stratton, introduced *BLOOD* – which is the *The Journal of Hematology. He was* then given the first post in the journal as the editor-in-chief.[27] BLOOD became the official ASH publication in 1976. His most important contribution was the creation of *American Society of Hematology* and *International Society of Hematology* in 1958 and 1946 respectively. William Dameshek held the post of the President of above said groups in 1964 and 1956, respectively.[28]

THE PHILADELPHIA CHROMOSOME DISCOVERY: 1960

Unexpectedly one day while working at the Pennsylvania University, Peter Nowell (1928), an biologist in tumor, noticed in Leukemia cases that the chomosomes at metaphases are visualized in cell culture slides[29], and in the same year Nowell with David Hungerford discovered Y Chromosomes. Chromosome spreading using hypotonic

solutions were discovered in 1953.[30] In 1960[31], the discovery of Nowell was published.

Nowell also established the specific chromosomal abnormality in classical 7 cases of CML.[32] This historic discovery followed the discovery of the specific chromosomal abnormality and finally the invention of the human chromosomes as diploid with 46 chromosomes in 1956[33]. This was followed by discovery of constitutional chromosomal abnormalities in 1959[34]. In CML, abnormally small chromosomes are discovered which are found to be directly disease related.

This chromosome in CML which was abnormal was termed as the Philadelphia chromosome (Ph^1) in 1960 where, for the first time, the international conference on Chromosomal Nomenclature was held. The concept that acquired chromosomal aberrations[34] are the cause for cancers was developed based on discovery of Ph chromosome.

CYTOGENETICS OF THE PH CHROMOSOME: 1973

In 1970, the techniques of chromosome banding using Giemsa and quinacrine methods by Caspersson and colleagues helped identify the Philadelphia chromosome.[35] The reciprocal translocation of Philadelphia chromosomes 9 and 22; involving the locus 34 and 11 was discovered as t(9;22)(q34;q11) by Janet Rowely in 1972.[36]

MOLECULAR DESCRIPTION OF *BCR–ABL: 1982 TO 1990*

ABL gene is mapped to chr-9 and is found to be 225 kb gene, which was discovered in 1982. In 1984, the same persons who discovered chromosomes also pointed out the breakpoint on chromosome 22 which is mapped to 5.8 kb area on long arm (q) of the chromosome which is termed as breakpoint cluster region and this breakpoint is found to be located in the 135 kb BCR gene.[37] Also 8.5 kb on chromosome 22 is termed as BCR-ABL fusion product in 1985 [38][39][40][41] that corresponds to 210 KD protein.[42] Later in 1986 & 1987, similar fusion was described in acute lymphoblastic leukemia with 190 KD protein product.[43] 210 KD protein was found to cause CML like disease in mice[44] and thus became the disease causing mutation in CML.

TARGETING LEUKEMIC STEM CELLS BY BREAKING THEIR DORMANCY:

Only a few population of tumor cells can form new tumors when they are transplanted to new host. This hierarchical organization of tumor cells is similar to self-renewing tissue and these cells which are termed tumor initiating cells or cancer stem cells (CSCs), that can give rise to new tumor and this hypothesis is first discovered in mice.[45] Like normal Hematopoeitic stem cells cancer stem cells show the same CD34+ CD38-

cell surface phenotype and their transplantation does not lead to proliferation of leukemia progeny.[48] In AML this initial observation was made and this same hierarchical organization was found in many leukemias. Like CML, many more solid cancers like breast, colon, prostate and brain tumors have this hierarchial organisation[47]. Based on different types of tumors, varying stages of the disease and individual parameters, the quantity of cancer stemcells vary. Cancer stem cells are very rare, in few tumors amounting to < 1 in 10 X 10^9 or < 1 in 100 X 10^9 cells, while in few malignancies, many cancer stem cells have tumor initializing capacity. Recent studies show that even differentiated tumor cells, either + or CD34-show tumor initiating properties as the nature of tumor becomes malignant[49].

The property of quiescence, high resistance to radiotherapy, DNA damage and repair mechanism and increased tolerance to anti-proliferative chemotherapy are seen in CSC as like normal stem cells. Because of the above said properties, CSC easily survive and they cannot be destroyed easily as like cancer cells in many solid tumors where debulking is easy. Tumor relapse and tumor metastasis is due to the presence of these residual cancer stem cells even though initial chemotherapy successfully destroys the tumor.

LEUKEMIA STEM CELLS IN CML:

Hierarchically organised leukemic stem cells are seen in CML after their initial description in Acute myeloid leukemia. As described previously, like normal hematopoietic stem cells, these leukemic stem cells in CML also have the same phenotype. But the phenotype of LSC in CML is still controversially discussed.

CML is characterised by overgrowth of bone marrow myeloid precursors and maturing series. It was the first malignancy that was linked to a specific genetic abnormality, a single chromosomal translocation between 9^{th} and 22^{nd} chromosome producing a fusion product whose chromosomal event (reciprocal translocation) being termed Philadelphia chromosome. Depending on the exact breakpoint the size of the resulting fusion protein varies, ranging from 185 to 210kD with p210 BCR-ABL being the clinically most important isoform [1]. The fusion protein BCR-ABL is the causative and is found to be sufficient for driving the development of the disease, and therefore became an ideal drug target. Thus came Imatinib mesylate, drug that specifically inhibits the BCR-ABL tyrosine kinase, as well as to a lower degree c-kit and PDGF-receptor.[51]

STEM CELL CONCEPT OF FORMATION OF CML:

Below are the evidences for single stem cell origin of CML:

a) Translation of all the myeloid series proliferation like erythroid, neutrophil, eosinophil, basophil, monocytoid and thrombocytoid proliferations in CML chronic stage.

b) The Philadelphia (Ph) chromosome could be demonstrated in all the above mentioned series[53].

c) A single G-6-PD isoenzyme could be demonstrated in all the myeloid series and red cells, but this is not seen in other somatic cells in patients with CML.

d) The occurence of the Ph translocation in a particular clone only, not in all cell lines.

e) The occurence of the Ph chromosome in single cell line only in patients who are sex chromosome mosaics.

f) Breakpoint within 5.8kb region of BCR-ABL gene among all cells, within a single CML patient, as described by molecular studies.

EPIDEMIOLOGY OF CHRONIC MYELOID LEUKEMIA:

Worldwide incidence rate of CML varies from as small as <1.0 to 2.0 per 1 lakh cases / Year, representing 14% of all cases of leukemia. Children harbouring CML constitutes about 3%, and in 5–20 years age group its 10%.[54] Switzerland, USA, Italy, Australia, Germany, and UK ranks highest in incidence rates, while Netherlands, Sweden, China, and India being the lowest.[55] More often affected are males when compared to females and the ratio is approximately 3:2.[56]

CLINICAL FEATURES OF CML:

Symptoms pertaining to anaemia, like fatigue, splenic discomfort, dyspnoea, weight loss and anoxia due to enlarging spleen are the most common symptoms. Neutropenia is absent and thrombocytopenia is not seen in chronic phase of disease. Most of the patients present asymptomatically, when blood tests are performed for routine check-up. Pallor and splenomegaly are the most common sign. 80-90% of cases have enlarging spleen. In absence of splenomegaly, a low leucocyte count of $<100 \times 10^9/l$ is noted. Increased leucocyte counts of more than 500000/l may be associated with dilatation of retinal veins. Diagnosis of CML can be made by blood counts and by peripheral blood films examination. Smear show increased proliferation of granulocytic forms, from myeloblasts to neutrophils, with two peaks consisting of mature

neutrophils and myelocytes, with few metamyelocytes. Leukocyte counts are markedly elevated from 250 x 10^9/l to even as high as 1000 x 10^9/ l on the time of diagnosis. Progressive rise in counts is seen in untreated patients.

Other findings include, reduced neutrophil alkaline phosphatase score, increased serum vitamin B12 levels and vitamin B12 binding protein which is transcobalamin 1. But these findings are not significant for the disease.

COURSE OF CHRONIC MYELOID LEUKEMIA:

Chronic myeloid leukemia evolves through three phases.

- Initial chronic phase
- Accelerated phase
- Blast phase

In the first chronic phase, patient presents with elevated total leucocyte count, as much as 80,000 cells/microL to as much as 100,000 cells . there may be slight reduction in haemoglobin concentration with marked elevation in platelet count. Invariably patients present with moderate to massive splenomegaly.[57] Patients respond very well to imatinib 400mg once daily. More than 90% achieve complete

haematological and cytogenetic response in three months of treatment. Few untreated or resistant cases or cases with abnormal cytogenetics go in for treatment failure and progress to accelerated phase.

According to revised World Health Organization (WHO) classification, evidence of "progressive disease" include features listed which are features of accelerated phase:

1) persistent or increasing WBC count of $>10 \times 10^9$/L with or without splenomegaly which is either stable or increasing progressively not responding to treatment;

2) persistent of thrombocytosis, with a count of platelet more than 1o lakhs/L not responding to treatment.

3) persistent of thrombocytopenia, with counts of platelets of less than 100×10^9/L not responding to treatment;

4) occurrence of cytogenetic non random chromosomal abnormality on progression independent of diagnostic cytogenetic study;

5) 20% or more basophils counted in blood from periphery

6) blood or marrow myeloblasts of 10 to 19%.[58]

"The WHO criteria for CML-BP include the following:

1) Blasts equal to or greater than 20% of the peripheral blood leukocytes or of the nucleated cells of the bone marrow or

2) An extramedullary proliferation of blasts."

Transformation of chronic to either accelerated or blast phase is characterised by following additional cytogenetic findings such as extra Ph, +8, +19 or isochromosome 17q in most cases.[59]

LABORATORY FINDINGS IN CML:

The peripheral blood complete analysis shows marked leucocytosis with median WBC count of > 100,000/μL [100×10^9/L]; with two peaks consisting of segmented neutrophils and of myelocytes.[60] Less than 2% of blasts ae seen, but absolute basophilia imay be present[61] in majority of the cases. The platelet count ranges from 1.5 to 30 lakhs/μl to more than 10,00,000 lakhs/L, but presenting with thrombocytopenia are very rare.

BONEMARROW ASPIRATION IN CML:

Increased cellularity & increased cellular fragments in marrow particles aspirated comprising of granulocytic proliferation together with a maturing cell pattern like that in blood and myeloblast count of 5% or less of the nucleated cells in the marrow.[62] Erythroid colonies are

decreased in number.[63] Size of the erythroid colonies are also reduced. Slightly there occurs mild decrease to marked increase in number of megakaryocytes. Megakaryocytes are small in number compared to normal and nuclei are hypolobated.

BONEMARROW BIOPSY IN CML:

In Bone marrow biopsy, chronic phase of Chronic Myelogenous Leukemia before treatment are characterized by a markedly heterogenous population demonstrable by trephine biopsies, immunohistochemistry and morphometry. Proliferation not only of granulocytic lineage, but also megakaryocytes, erythroid precursors, and lymphocytes. Stromal components composed of reticulin and collagen fibers has a major role in the disease process. Number of megakaryocytes and number of their precursors have correlation with fiber density. Thus in pathomechanisms of myelofibrosis megakaryopoiesis and fibroblasts have functional relationship. In 30% cases, reticulin fibrosis is shown to be moderate to marked at diagnosis, demonstrable with reticulin stain in biopsy samples and this feature is associated with a poor prognosis in spite of tyrosine kinase inhibitors. Also there is correlation between increase in stromal component and features like anemia, spleen size percentages of myelobasts and erythroblasts in the peripheral blood or LDH level. These features are seen in advanced stages of CML which implies a transition to

myeloid metaplasia and carry a significant prognostic impact on survival.[65]

SPLEEN AND CML:

Splenomegaly is present in invariably all patients with chronic myeloid leukemia irrespective of phase of presentation. Size of the spleen correlates with prognosis in cml.[65] In cases that present with CML blast crisis, presence of splenomegaly is an important sign to differentiate these patients from denovo AML, ALL from CML myeloid & lymphoid blast crisis.

DIAGNOSTIC MOLECULAR MODALITIES IN CML:

There are 3 phases in study of cytogenetic:

Three phases in Cytogenetics of human cells are:

i) The first phase:

Methods required to produce metaphase spreads. Skin fibroblasts are used initially in 1956 for cell culture. After 40 years, phytohemagglutinin was identified, which could stimulate lymphocytes particularly T cells in blood and was found to stimulate mitosis. Thus this analysis made cytogenetic analysis, a diagnostic tool from a research method. Finally in 1960, the Philadelphia chromosome was the chromosomal abnormality that was associated first with Chronic Myeloid

Leukemia. Later, upto 1969, Cytogenetic study was performed with stain like giemsa, which stains the chromosomes uniformly throughout the length. So, defining became difficult when entire chromosomes were stained and were of similar shape and size. So in 1960, a nomenclature was framed that groups chromosome depending on size, at the Conference of Denver, which was later modified in 1967 at the Chicago Conference.

(ii) The second phase:

In 1969 to 1971 chromosome banding techniques were discovered that paved way for second phase of cytogenetics in malignancy. In 1971, at Paris Conference, the convention used for numbering of each identified chromosome and banding of them was established. In 1972, first translocation of chromosomes 8 and 21 [t(8;21)(q22;q22)] was identified in AML. It was enumerated that the cytogenetic abnormality Ph chromosome was identified to be due to reciprocal translocation involving 9 and 22 chromosome [t(9;22)(q34;q11)][1] and not due to deletion was found. Proto-oncogenes are associated with breakpoints on the chromosomes. So likely, the breakages cause normally functioning genes to be disrupted. Thus ACA carry diagnostic and prognostic significance.

(iii) The third stage:

Stage where chromosome regions/genes are identified by the use of specific DNA probes. With the introduction of fluorochrome probes that paint specifically different human chromosome and probes that fluoresce different genes, many abnormalities are detected. This is called by Fluorescence InSitu Hybridization (FISH) technology. Chromosomal losses and gains are identified by Comparative genomic hybridization (CGH). All chromosome defects are detected by multicolour FISH or spectral karyotype methods. With this only the chromosome involved are detected but specific region affected in chromosome are not detected. Use of Rx-FISH techniques and DNA labelled with flours helps to improve the precision of karyotyping.[66] Cytogenetic studies is also used to type the hematological malignancies and to monitor the disease progression, like presence of ACA in CML developed on disease progression in cases who had only a Ph chromosome at the time of diagnosis indicates progress to acceleration / blast crisis. These studies are also valuable tool to assess the success of marrow transplant and impact of chemo on marrow. Because in remission after chemotherapy with PTKI a normal karyotype is observed. Replacement of abnormal karyotype by donor karyotype indicates engraftment after BMT. Prediction of the disease prognosis can

also be made based on the cytogenetic abnormality observed as in case of AML, M2 t (8, 21) indicates a good prognosis.

CYTOGENETIC PROCEDURES

Conventional cytogenetic studies need actively replicating cells that are usually obtained in marrow of bones. Highly proliferating cells are also seen in lymph nodes, chorionic villi and solid tissues. When peripheral blood lymphocytes are cultured, mitotic stimulant like phytohemeagglation has to be added to the cells to make them undergo mitosis. After capturing the metaphase spread, banding is done with giemsa and chromosomes are distinguished individually by observing under ordinary light microscope. Usually the specimen choice is based on clinical indications of the patient for the chromosome analysis. Presence of hematological malignancies like lymphoma or acute or chronic leukemias, the marrow aspirate is the specimen used.

A) COLLECTION OF SPECIMEN

Sodium heparin tubes are used for collection of bone marrow specimen which is preservative-free. First few mm of tap from marrow have the maximum proportion of cells and they are samples considered the best for cytogenetic analysis. Later bone marrow taps are diluted by blood and this decreases the number of actively dividing cells. The

number of actively dividing cells determines the success of BM. After harvesting, transport specimen to lab immediately at room temperature or in case of delay that which could not be denied, the specimen should be stored at 4^0 C and must be transported in an ice packed vacuum flask. Specimens are processed upon reaching lab at once to avoid death of cells.

B) CULTURE MEDIA

Samples for cytogenetic studies are cultured in aqueous media like RPMI 1640 for good growth. Thus media are solutions of balanced salts and are composed of salts, glucose, and buffers to maintain the correct pH. These media that are commercially obtained. But still they have to be cultured together with antibiotics L-glutamine and fetal bovine serum (FBS). As bone marrow contains actively dividing cells, for BM culture, phytomeagglution is not added. The amino acid L-Glutamine is for cell growth but it is not stable and it breaks down on storing to form D-glutamine. Its isomer L-glutamine should be kept frozen to maintain its stability. So it should be put into the culture medium just before its use. Also required is Serum for better cell growth. Fetal bovine serum of 10-30% of serum is included into the culture medium. Antibiotics like Penicillin/streptomycin, gentamicin, and kanamycin are used commonly in culture media to stop the growth of microorganisms.

Phytohemeagglutinin, a mitotic stimulant is not applied for bone marrow. But, B-cell mitogens like Epstein Barr virus, are used to stimulate lymphocytes in case of B-cell lymphoma and many leukemia. Free floating cells in blood and bone marrow samples are cultured in tissue flasks or sterile tubes.

C) SPECIMEN PREPARATION FOR CULTURE

Either the sample like marrow or blood should be directly added to the culture or WBC separation should be made from whole blood and it can be useful to inoculate the culture. White Blood cells are separated by keeping it in rest or by doing centrifuge of the sample. Wait till the blood in test tube settles, it will be settled into 3 unique layers. The heavier RBC settles in the lowest layer, plasma tops the most upper layer, and the the buffy coat layer i.e the middle layer consists of the WBC. Take away the layer of buffy coat and resuspend using the culture. Culture the marrow cells at 37^0C using an incubator. It can be harvested directly also, by spending no time in culture, or after giving 24 hours to 48 hours culture time for increasing the mitotic index level. Extended duration in media are not need, because the cancer cells which are abnornal can be lost over the time or normal precursor cells may be diluted which is present in.

D) HARVESTING CELLS

Once the cultures are grown for prescribed duration of time and sufficient counts of dividing cells are there, the harvesting of cells can be done. While harvesting the first step include the collecting of dividing cells that are in metaphase, followed by treatment using hypotonic media, fixation and the placing the chromosomes on the glass slides, to stain and examine microscopically. A inhibitor i.e mitotic, colcemid is analogue of the drug colchicine which is normally suggested and used in order to attract enough count of cells at the metaphase. The tubulin is binded by the Colcemid, which is a protein, obstructing the formulation of the spindle fibers or removing the spindle fibres already there. Thus avoids the separation i.e the sister chromatids' separation in anaphase, favours grouping of cells at metaphase. The essential step is the exposure time to colcemid. The longer the duration higher the metaphases is collected. Actually they are shorter, because of the condensation of the chromosomes on progression through the metaphase. Longer chromosomes should be taken for studies. After exposing cells to colcemid, the hypotonic solution is added. Water enters the cells by osmosis, results in cell swelling. This step is very important for enough chromosomes spreading on the glass slide. Timing is the very crucial factor. Cells bursting may be caused by the longer duration of exposure

and similarly chromosomes clumping may be caused by shorter duration of exposure. Some hypotonic solutions can be useful, namely: 0.075M potassium chloride (KCl), 0.8% sodium citrate, dilute serum, diluted balanced salt solutions, and mixtures of KCl and sodium citrate. The type of hypotonic solutions we use affects the chromosomes morphology. Fixative containing or consists of 3 parts of absolute methanol and one part of glacial acetic acid stops the hypotonic solution action and also fixing the cells at the swollen stage. The modified carnoy's lyses the rbc in the sample. Every time fresh fixative must be prepared before it can be used because it can get diluted by absorbing water in the atmosphere. The fixed cell suspensions are dropped on the glass slides. And the concentration of suspension is adjusted to get most favourable results.

A high-quality slide grounding must have:

- adequate quantity of metaphases. Cells should not be packed out on the slide,
- Metaphases that are spread well with minimal chromosomes overlap,
- No observable cytoplasm.

Elevation of high temperature and moisture enhances the chromosomes distribution, whereas cooler, reduced temperature and

lesser humidity decreases the chromosomes spreading. Higher duration exposure to treatment of hypotonic media makes the cell more easily broken and also increases the spreading. But an insufficient exposure to hypotonic solution makes the cells difficult to burst.

Variables during the slide preparation include:-

- height from where the cells are dropped;
- use of wet slides or dry slides;
- use of cold slides, room temperature slides, or warm slides;
- use of air or flame or steam drying of the slides; and
- the angles at which the slide is spread and/or pipette is held.

The slides should 'aged' overnight at 60^0C or at 90^0C for 1 hour to enhance chromosome banding. It can also done by brief exposure of the slides to UV light[67].

E) BANDING AND STAINING OF CHROMOSOME

Techniques of Chromosome staining and colour banding are of 2 broad categories:

i) First technique produces specific bands that alternate throughout the length of the chromosome, and

ii) Second technique, stains only a particular region of some chromosomes or all of the chromosomes.

Techniques that produce banding along the length of each chromosomes include:

- Giemsa banding,
- Quinacrine banding and
- Reverse banding.

This technique helps in positively identify each of the chromosome pairs and it also permits the categorization of the structural abnormalities. Resolution of banding is an account of the consecutive light /dark bands number in a haploid set. The minimum band resolution is 400 bands. Good banded, moderately increased resolution metaphase have 500-550 band range, and prometaphase have a resolution of exactly 850 or more bands range.

GIEMSA BANDING:

G-banding is the most commonly used routine banding system. G bands formed with trypsin and stain like Giemsa or GTG banding is a banded techniques among many. The slides that are aged are treated with enzyme trypsin and stained with Giemsa. Other than Giemsa stain, others like Wright or Leishman stain are also involved. A light/ dark band series

are also produced which helps in the notification of each chromosome. The dark band that are A-T rich, with heterochromatic introns in the chromatid, whereas the bands that are light have G-C rich areas, with euchromatic regions. The most active regions are G-light bands compared to the dark bands which have comparatively minimal number of active genes.

Q-BANDING:

This fluorescent technique is like G-banding pattern but with very few exceptions. The large pericentromeric and polymorphic regions of chromosomes 1 and chromosome 16, and the end of long arm (q) of the chromosome Y which brightly fluoresce. The end of long arm of chromosome Y is the site with most fluorescent activity in the genome of human. In the advantage of Q-banding is that, it confirms the presence of Y , but the negative advantage is that this is not permanent, as it is a fluorescent stain and require the use of fluorescence microscopes which are most expensive and a dark room.

R-BANDING:

The technique of R-banding creates a banding pattern which is reverse or opposite pattern of G-banding. Available are fluorescent and methods that are nonfluorescent. Human chromosomes inherit

euchromatic terminal ends or telomeres that are difficult to vizualise with standard Giemsa band techniques as the telomeres which are pale regions on chromosome ends may hide into the background. R-banding helps in identification of these telomere.

Methods that stain selective regions in chromosome include:

- Constitutive heterochromatin staining
- Telomere banding or T-banding
- Cd staining
- Giemsa at pH 11 or G-11 banding,
- Silver staining for nucleolar organizer regions or NOR staining and
- 4,6Diamino-Phenole-Indole/Distamycin A or DAPI/DA staining.

C-BANDING:

It is a technique that particularly stain the constitutive heterochromatin located among the centromeres, the regions of inherited variations located on chromosomes 1, chromosome 9, chromosome 16, and the end of long arm (q) of the chromosome Y. This banding is helpful for studying marker chromosomes and determining the presence of pseudocentric and dicentric chromosomes.

TELOMERE BANDING:

Telomere banding helps in locating only the terminal ends or telomeres of the chromosome stained.

CD STAINING:

CD staining procedure produces a dot pair one for each chromatid at each centromere. Only the functionally active centromeres will be stained. This staining is used to classify functional from not functioning centromeres and to study the presence of translocations, to locate ring chromosomes and to study marker chromosomes.

G11-BANDING:

This banding technique particularly stains the heterochromatin areas of chromosomes 1, chromosome 9, , chromosome 16, the end of long arm of chromosome Y, and the satellites regions of the involving acrocentric chromosomes. G -11 banding is also used to classify between human and other chromosomes in hybrid cells.

NOR STAINING or AgNOR:

AgNOR staining particularly stains the nucleolar organising regions located on the stalks of the every acrocentric chromosomes. Silver staining of nucleolar organising regions stains particularly the active ribosomal genes. This silver staining of nucleolar organising

regions helps to identify the marker chromosomes and to study the rearrangements or variations involving every acrocentric chromosomes.

DAPI/DA STAINING:

This staining combines, a fluorescent dye, along with distamycin A, which is not a fluorescent antibiotic. These **DAPI/DA** create stable bonds like A-T rich areas and double stranded regions of DNA. DAPI/DA fluoresce few A-T rich regions of constitutive heterochromatin in the band region c of every chromosomes 1, chromosome 9 , chromosome 16, and end of long arm of Y chromosome, and the short arm (p) of chromosome 15. This method is used to find rearrangements of chromosomes 15 and to confirm variations over polymorphic areas of chromosomes 1, chromosome 9, chromosome 16, and end of long arm of chromosome Y and to find marker chromosomes.

CYTOGENETICS – AUTOMATION:

Cytogenetic methods are quite difficult and depend on man power. Instruments such as robots that harvest, controlled drying units, and imaging systems which are computerized have been designed to assist the lab in sample preparation and identification of chromosomes. Some laboratories doing cytogenetic studies use all the above said automations, some use few, and some never use any methods.

A) ROBOTIC HARVESTERS

These harvesters do harvesting of actively dividing mitotic cells for cytogenetics after the addition of mitotic inhibitor colcemid which produce metaphase arrest. The robotic machine can perform aspiration, removal and taking in of hypotonic solution, and as well as fixing of cells. The incubation times should be programmed within computer for each step that runs the robot.

B) DRYING CHAMBERS

Helps in spreading chromosomes by keeping one or more drops of above said suspension of cells on slides. Metaphase spread is controlled by height of fall of the suspension, procedure temperature and the conditioning of the spread, air flow and humidity of the surrounding room. cell suspension dropped over the slides dries and the fixative has pulling action on the cell membrane over the slide, making the metaphase cells to spread across. There are commercialy available Conditioned controlled units where air flow, humidity and temperature is maintained and can be maintained for slide preparation.

C) COMPUTERIZED IMAGING

The traditional method of cytogenetics involve the principle of photo capturing from microscope after the location of appropriate

metaphases by use of an England finder. Camera is attached over the microscope to take pictures of spread of chromosomes. The film is processed in room that is dark. The cell spreads with metaphase are paired after cutting, but this procedure consumes lot of time. This process can now be done using a computerized imaging method. The metaphase spreads can be captured by image acquisition subsystem. The subsystem consists of a camera over microscope with an adapter, CCD, a grabber for frame and an expensive image capture software. Dedicated imaging software can do either automatical or semi-automatical karyotyping, banding analysis and ideogram display which are all now available commercially. Components like metaphase finding, multicolour FISH analysis, Comparitive Genomic Hybridization analysis can be included. Captured metaphase spreads in the digital form are analysed by the Cytovision satellite capture station. This image is made to transfer into the Cytovision workstation where karyotyping method is performed. Karyotyping in this method is done semi-automatically. The manual checking by geneticists should be done to assure that the chromosomes are correctly arranged and paired. A hardcopy is produced by laser printer consisting of the metaphase spread and karyotype. The metaphase spreads and karyotypic patterns are stored in the form of files on the optical disks.[68]

A) CYTOGENETIC ANALYSIS REQUIREMENTS FOR NEOPLASTIC STUDIES

Cytogenetic analysis can be done from marrow aspirate of long bones or ileum, peripheral blood that is not stimulated or from biopsy of tumor or aspirate. Cells are karyotyped according to the ISCN, 2013. Complete karyotyping of 25 cells are done and all 25 metaphase spreads are analysed. At least two karyotypes should be prepared from each cell line if >one cell line is present with a resolution of at least 400 bands. If there is <20 analysable cell plates with an abnormality detected, the number of normal and abnormal cells (if any) is detected. If there is less than 20 examined cells and if abnormality is not been detected, the number of cells studied is reported and many additional procedures like FISH and molecular studies could be done if clinical condition warrrants. Additional metaphases may be examined for studies of minimal residual disease or marrow transplant.

B) FAILURE OF CULTURE

Failure of Bone marrow culture may be due to:

i) the specimen submitted to the laboratory is inppropriate, which include blood drawn from periphery with no blasts collected

instead of marrow. This may be the result of the wrong specimen tube being collected.

ii) specimen submitted to the Laboratory is insufficient

iii) no living cells in the sample due to delay in sample transportation or improper storage of sample, that is sample not kept at 4^0C and not transferred in ice if delay is not avoidable.

iv) catastrophic equipment failure in laboratory.

v) Reagent worn out.

vi) interpretation error (most unlikely).

CYTOGENETIC ABNORMALITIES IN CML:

In patients with chronic myelogenous leukaemia (CML), the break points are heteromorphic or polymorphic on the long arm of chromosome 22 (22q) producing the Philadelphia (Ph1) chromosome which is heteromorphic in size for the long arm of chromosome 22. Four types of Philadelphia chromosomes are proposed based upon the break points and with the relative size of chromosome. They are: Types I (very large), type II (large), type III (average) and type IV (small) with respective break points at bands 22q13.3, 22q13.1, 22q12 and 22q11.3, respectively. Since the break points are considered arbitrary and hence these types are not considered absolute as they are based on length differences. Since types I

and II are difficult to recognize by conventional techniques, the RFA technique that is Reverse band by fluorescence with acridine orange should be performed on all cases.[69] Addition to the most common and characteristic Philadelphia (Ph) chromosome t(9;22)(q34;q11), the frequency of ACA, additional chromosomal abnormalities has an incidence of 7% in chronic phase and increases to 40–70% in advanced disease/blast crisis.[70][71] Progression from chronic phase to accelerated phase or blast crisis is often associated with additional chromosomal aberrations like trisomy 8, trisomy 19, duplication of the Ph chromosome, monosomy 7, acquisition of t(1;21), isochromosome 17q (leading to the loss of p53 gene on 17p), or inversions and translocations associated with AML/myelodysplasia[72], which translates a genomic instability of cells and the appearance of *BCR-ABL1* kinase mutations, both of which can confer resistance to tyrosine kinase inhibitors.[73][74] Very few cases of lineage switch in CML have been reported in the literature before[75] and during the imatinib era[76]. The karyotype analysis showed that the chromosomal aberrations occurring in addition to the Philadelphia chromosome (Ph^1) were non-random strictly. In a study, 88% of the total cases with further changes, at least any one of the three main chromosomal aberrations wasdetected. An extra Philadelphia, trisomy 8 and/or trisomy for the long arm of chromosome 17 were

observed in all cases studied.[77] Variant translocations occur in 5%-10% of patients, and [t(v;22)] are characterized by the involvement of one or more chromosomes in addition to chromosomes 9 and 22.[78] On treatment with imatinib, patients with variant translocations and standard translocations standard translocations had a similar prognosis.[79] Patients with variant translocations have deletions in derivative chromosome 9 [der(9)], that were a key prognostic factor in the pre-imatinib era, occurring mostly in t(v;22) patients.[80] The loss of the negative prognostic impact of der(9) deletions with imatinib contributes to the fact that variant translocations does not have negative prognosis.[81] Near haploid leukemia stemlines were also reported[82]. Other than the additional chromosomal abnormality, disease progression is found to associate with marker chromosomes. Multicolour FISH studies has enabled the detection of the marker chromosomes[83]. Correlation among the type of additional chromosomal aberration along with the phenotype of blast crisis has been established. Hyperdiploidy, trisomy 8, trisomy 19, ,trisomy 21 and isochromosome(17q) are linked with myeloid blast crisis. An extra Philadelphia is seen both in both myeloid and lymphoid crisis. Chromosome loss is rarely seen compared to chromosome gain in blast crisis. If loss is seen, monosomy 7 is the most common. They precede the blast crisis by 2-4 months. Especially in an upcoming blast

crisis, following up with cytogenetics would be a valuable measure for monitoring of the progression of course of disease[1].

VARIANT PHILADELPHIA TRANSLOCATIONS:

Variant translocations occur in 5%-10% of patients, i.e, [t(v;22)], are considered by the involvement of one or more chromosomes along with chromosomes 9 and 22.[84] The mechanisms of the creation of the variant translocations are not clear; some have optioned two different mechanisms of origin: one step mechanism in which chromosome break is occuring instantaneously on 3 or 4 different chromosomes in a 3 or 4-way changes, respectively, and a two step mechanism involving subsequential translocations in which a standard Philadelphia t(9;22) translocation is followed by another translocation involving additional chromosomes.[85] The 2-step mechanism suggests that the variant translocation is like clonal evolution, and thus this mechanism could be an association with a poorer prognosis.[86] The mechanism of variant translocations can be detected by FISH technique used at present. In the era prior to imatinib, some studies reported a strong association of variant translocations with the presence of der(9) deletions,but this is disproved[87][88][89] suggesting a poor prognosis which may be due to the increased frequency of the der(9) deletion changes.

PHILADELPHIA NEGATIVE CML:

More than 90% of patients diagnosed with a morphologic picture of chronic myeloid leukemia (CML) demonstrate the characteristic reciprocal Philadelphia chromosome (Ph) karyotypic abnormality, t(9;22), by conventional cytogenetic analysis.[90] The remaining 10% of patients are classified into Ph negative CML or ph with additional chromosomal abnormality by conventional cytogenetic method of karyotyping. In these cases, the bcr/abl gene rearrangement is found out by molecular studies in 25–50%. Patients in this group are classified with Ph negative bcr/abl positive CML.

Studies identified a well characterized group of patients with Philadelphia negative, bcr/abl negative CML. This group represents 2.9% of all patients with CML who were seen at The University of Texas M. D. Anderson Cancer Centre between 1967 and 1999.[91] A median survival of 25 months is seen in Patients with bcr/abl negative CML from the time of diagnosis.[92] in another study the median survival of 50 months is seen in patients with Ph negative, bcr/abl positive CML, with a survival similar to that has been reported for patients with Ph positive CML.[93] These finding provide further support, that Ph negative, bcr/abl positive CML is indistinguishable clinically and biologically from Ph positive CML.[94] adverse features assoiated with survival are Older age, low platelet count,

low haemoglobin(anemia), and increased percentages of blasts in eripheral blood. By multivariate analysis, older age, anemia, and severe leukocytosis as variables that were associated independently with poorer prognosis. Diploid karyotype is seen in two thirds of patients. Trisomy 8 was the most common abnormal karyotype observed in cases with additional abnormality. Frequency of additional chromosomal abnormalities was about 30% in both the bcr/abl negative group and the bcr/abl unknown group. Similar reports are obtained from the previous studies.[95]

FISH ANALYSIS (FLUORESCENCE INSITU HYBRIDIZATION):

This procedure is done on bone marrow cells using DNA probes that hybridize at the BCR and ABL regions prepared by the standard cytogenetic techniques . The most frequently used probes were: Dual-Fusion Translocation Probe (Abbott Molecular-Vysis), the Locus Specific Identifier BCR/ABL Dual-Color, or the Double-Fusion Signal D-FISH BCR/ABL Probe. Probes for ABL and BCR gene regions uses fusion strategy in dual fusion technique. A normal nucleus will exhibit 2 orange and 2 green signal patterns; one orange (normal 9 chromosome), one green (normal 22 chromosome) in the nucleus is seen with a classic t(9;22) translocation, and derivative chromosomes der(9) and der(22) show 2 yellow fusion signals in the nucleus.[96] The Locus Specific

Identifier BCR/ABL Extra-Signal Dual-Color Translocation Probe uses an extra signal. The nucleus with a classic Philadelphia t(9;22) translocation will display 2 orange signals on normal chromosome 9 and der(9), and one fusion signal yellow on der(22).

Hybridization pattern varies in cases with structural changes involving chromosomes 9 and 22 or with a variant Philadelphia translocation. Slight modifications were done for Hybridization procedures according to the manufacturer's recommendation. A pool of PAC, RP5-1132H12, and RP5-835J22 known as PAC/BAC system is used in only one laboratory for the ABL gene and for the BCR gene, BAC and RP11-164n13 is used.[97] 200-300 nuclei on metaphases are analysed in FISH analysis to localize the rearrangements.

MOLECULAR GENETICS OF CHRONIC MYELOID LEUKEMIA:

INTRODUCTION TO MOLECULAR STUDIES

In B-cell or T-cell lymphoproliferative disease, evidence of clonality for gene rearrangement can be studied by Molecular studies. This also helps to identify certain chromosomal translocations associated with the leukemias. Other techniques for gene rearrangement are Southern blot hybridization, pulse gel electrophoresis and polymerase

chain reaction (PCR). For detecting the *bcr-abl* gene rearrangement in CML, polymerase chain reaction is used for the detection of nature of transcripts, for the presence of the b2a2 and b3a2 transcripts. The presence of one of the b2a2 or b3a2 transcripts indicate that the patient has CML.

POLYMERASE CHAIN REACTION (PCR):

A) INTRODUCTION TO THE PCR

Kary Mullis is the person who invented polymerase chain reaction (PCR) in 1985.[98] principle pf PCR is to amplify a small segment of DNA over a billionfold. The substrate used in PCR are a pair of 15 to 50 oligonucleotide primers, DNA polymerase, deoxyribonucleotide triphosphates (dNTPs) , and buffer. oligonucleotide primers are sequences with a length of 15 to 50 oligonucleotides, which are synthesized against opposite strands of DNA that has to be hybridized several kb apart. The oligonucleotides prime the synthesis of DNA on the DNA template. Each cycle in PCR consists of a set of 3 steps which are repeated under different temperature controlled conditions in succession.

I) HEAT DENATURATION

The first step is to dissociate (double stranded) ds DNA which is a template into two strands by means of heating at high temperature.

II) ANNEALING OF PRIMERS

On lowering the temperature, two DNA strands reassociate forming primer-template complex as excess of oligonucleotide primers are present when compared to DNA templates.

III) PRIMER EXTENSION (AMPLIFICATION STEP)

The third step is the formation of complementary DNA where the oligonucleotides attach to the extending end of the primer DNA by use of DNA polymerase. The extension occurs from 5'to 3' direction of each annealed primer on the complementary DNA (c-DNA) strand.

These 3 steps completes one entire cycle. Generally in PCR, 25 to 30 or more cycles are performed using the newly synthesized DNA fragments acting as template for the synthesis of new DNA strand in next cycle. Thus for the new DNA synthesis the fragments of DNA synthesized in the previous cycles becomes templates. These 3 steps can be automatically done using a thermal cycler. The amount of DNA fragments formed in each cycle increase exponentially at a rate of 2^n, where n equals the number of cycles performed. Products formed in the PCR consists of DNA fragments with lengths that are uniform in all fragments. These DNA fragments are the products of oligonucleotide priming. Amplicon or target sequence are products with nucleotide

sequences that are complementary to oligonucleotide primers. However, the exponential amplification is not limited process but continues uninterrupted. Efficiency of each cycle in PCR is governed by number of factors.

Factors are :

- The amount of enzymes
- The amount of substrates added
- Temperature conditions.

Effect of these factors is more pronounced in later cyles of PCR. The enzymes are exhausted after 2530 cycles of PCR due to production of excess amounts of target. The activity of the enzymes are reduced due to thermal denaturation. Reannealing of target strands occurs when target strands concentration increases. This competent increase of target strands competes with primer annealing[99]. The effectivenss of PCR is such that it can amplify the DNA fragments that are very small as much as 5 kb.

But the disadvantage is that PCR is not too good for amplifying larger fragments of DNA. In case of large DNA fragments, there is difficulty of synthesizing DNA of sufficient length from one primer and to make that primer strand to serve as a template strand for the other primer in the next amplification cycle.

Uses of PCR include:

- Disease diagnosis
- identification of species
- detection of viral or bacterial infection
- prenatal diagnosis
- forensic sciences

B) COMPONENTS OF THE PCR

The basic components used in PCR include the following:

- DNA polymerase
- DNA template
- dNTPs
- primers,
- magnesium chloride (MgCl), and
- buffer.

  Some come in kit form.

(I) DNA TEMPLATE

Two DNA template are usually provided by the researcher or clinician. In general, lengthy DNA template sample preparations are not required for the PCR to work efficiently. Even crude DNA samples can

be used as templates as the technique of PCR is highly sensitive. simple boiling itself will help in extracting DNA from samples like paraffin embedded tissue block sections or ancient samples are also used in PCR. As only short intact nucleotide sequences are required for PCR, however crude the DNA samples may be, they can be analysed in PCR.

(II) PRIMERS

Required Primers for each case can be prepared from preformed genetic sequences which are available from sources such as EMBL OR GENEBANK. These genetic sources have nucleotide sequences which are evolutionary conserved sequences or they contain sequences based on existing sequences of close similarities. Primers can also be designed from already known information of sequences of amino acid. Features of primer that affect the PCR are the composition of primer, which must contain sequences that atleast match 50% of template sequence and the ability of the primer to form primer-dimer structures or that it should be complementary to itself. Both of these factors affect the PCR.[100] Many computer programmes are available currently to aid in the process of designing primer and optimization of annealing temperatures.

(III) THERMOSTABLE DNA POLYMERASE

DNA polymerase 1 with its Klenow fragment is used in the initial development of the PCR. DNA polymerase 1 is the enzyme used to carry out the extension step of the PCR. It is a heat labile enzyme. Because of its lability to heat, during every step of PCR, enzyme should be added freshly as the enzyme is denatured by the high denaturation temperature. This step of addition of enzyme in each step incurred more cost and made the technique labour intensive. But this difficulty is overcome by the thermostable DNA polymerases. These DNA polymerases which are thermostable made PCR a fully automated technique as the enzyme needed to be added only once. The *Taq* DNA polymerase is the most commonly used first thermostable DNA polymerase. It is produced from bacterium *Thermus aquaticus* (*Taq*) which is found in the hot springs of Yellowstone National Park. *Taq* and recombinant form of taq which is Amplitaq have 5'-3' exonuclease activity. It is stable at an optimum temperature at 72^0 C. These two complementary DNA polymerase lacks 3'–5' proofreading exonuclease activity. This property made it as the cause for wrong incorporation of nucleotides.

The Stoffel fragment DNA polymerase, derivative of taq DNA polymerase enzyme, is highly thermostable and is less sensitive to changes in Magnesium concentration. The Stoffel fragment DNA

polymerase, has no 5' – 3' exonuclease activity. Stoffel fragment polymerase is aids in amplifying G-C rich regions. Since G – C rich regions require high or prolonged temperatures, thermostable Stoffel fragment DNA polymerase are required. There are numerous DNA polymerases available and marketed commercially. highly thermostable DNA polymerases known as Vent^{TM} DNA polymerases is isolated from the bacterium *Thermococcus litoralis.* These bacteria are found in the floors of deep oceans and have capacity to extend templates in excess of 12 kb pairs. These DNA polymerases have higher fidelity and 3' – 5' proof reading ability when compared to *Taq* DNA polymerases. A derivative of the bacterium *Thermococcus litoralis* lacking the activity of exonuclease enzyme which is 3' – 5' proof reading activity is also available known as Vent (exo -)^{TM}. Also a form of DNA polymerases with very high thermostability known as (Deep Vent^{TM}) are also commercially available.

Hyperthermophilic variant which is a marine derivative synthesized from the hyperthermophilic archaebacterium *Pyrococcus furiosus* (*pfu*).this *Pfu* DNA polymerase also contain 3'-5' proof reading activity and incorporates radiolabeled analogues and nucleotides efficiently. *Pfu* DNA polymerase is very useful for producing radiolabeled gene probes. *Pfu* DNA polymerase also helps in cycle

sequencing techniques. However, degradation and modification of primers and template can be caused by the 3' – 5' exonuclease activity of *Pfu* DNA polymerase. Hence, this enzyme must be added last when preparing the reaction. A mutant clone of *Pfu* DNA Polymerase called *Pfu* DNA Polymerase (exo-) which is a genetically engineered product has no 3' – 5' exonuclease proof reading ability is also commercially available. A recombinant form of DNA polymerase and its modified form obtained from *Thermotoga maritima* (*Tma*) from the DNA polymerase gene of Thermotoga maritima called as UITma^{TM} DNA polymerase is available. *Tma* is a hyperthermophilic, Gramnegative true bacterium. Thermotoga maritime was isolated by heating the marine sediments geothermally in Italy.

A DNA polymerase with reverse transcription activity known as *Tth* DNA polymerase is available. It is formed from *Thermus thermophilus* (*Tth*), which could perform reverse transcription reactions in the presence of Mn^{2+} at 70 ^{0}C. After chelation of Mn^{2+} and DNA synthesis, the polymerase can carry out polymerization of the template. This dual activity of reverse transcription reactions and DNA synthesis has made the *Tth* DNA polymerase as a best choice for RT-PCR to be carried out in a single tube.[101]

IV) OTHER COMPONENTS OF THE PCR:

The DNA polymerase requires $MgCl_2$ for efficient activity and in the extension step of the PCR for incorporation of dNucleotide Triphosphates. $MgCl_2$ concentration also affects the specificity of the DNA template and primer interaction. $MgCl_2$ concentration increasing the melting temperature thereby affecting the denaturation of the double-stranded DNA template. Concentration of $MgCl_2$ is important in such a way that low yields are due to Insufficient Mg^{2+} Concentration whereas non specific products are formed when concentration of $MgCl_2$ is in excess. pH has to be maintained at 8.3 at room temperature. This is achieved by the buffer/salt composition. 50mM KCl and 10mM Tris-HCl are the usual components of the buffer/salt composition. changes in temperature cause significant changes in pH when tris-HCl is used as buffer. This Ph change may especially affect the amplification of long fragments[102].

However $MgCl_2$ and PCR buffers such as 50mM KCl and 10mM Tris-HCl can also be prepared manually depending on the requirements of the sample load in each laboratory. Commercially available kits contain DNA polymerase, buffers and $Mgcl_2$ together.

C) HOT START

When primers that are degenerating are used mispriming of oligomerization of DNA occurs. 'Hot start' technique been used to increase specificity of the polymerase chain reaction. By this technique the separation of one or more of the important reagents of the PCR occurs. nonspecific annealing of primers are prevented by using hot start technique. Hot start technique helps in mixing of all reaction components after the DNA template are denaturation. PCR using the hot start technique minimizes to nontarget DNA sequences and decreases primer oligomerization.

D) CONFIRMATION OF PCR PRODUCTS

Agarose gel electrophoresis helps in confirmation of the amplified PCR products. Restriction endonucleases can also be used for confirmation of PCR products. These restriction endonucleases works based on the presence of a appropriate restriction site within the amplified sequence. A DNA binding fluorescent dye is used to stain the gel. The fluorescent dye used is ethidium bromide which stains the dye and results are viewed under ultraviolet light[103]. Southern Blot analysis also helps to confirm the amplified PCR products. Following this technique hybridization with appropriate DNA probes are done.

Second round of PCR can be done on products that are produced by amplification of DNA fragments in first round of PCR. This second round can be done by nested PCR technique whereby nested oligonucleotide primers that corresponds to the sequences that are present within the amplified DNA sequence is used.

False-positive reactions in PCR may be due to:

- False priming either on the genomic DNA or on the primers themselves can occur. Hence irrelevant DNA fragments are formed on amplified fragments.

E) CONTAMINATION PROBLEMS OF PCR

As the technique of PCR is highly sensitive technique, even minute contamination may cause amplification of wrong DNA fragments. care taken should be utmost to avoid contamination. Potential sources that cause gross contamination are DNA from previously amplified products or DNA included during sample preparation. Contaminating DNA fragments from previously amplified products are found on pipettes, laboratory surfaces and even aerosols. Inorder to avoid such gross contaminations, units where reactions of PCR are done should be set up in a separate sterile locations within a laboratory.

DNA extraction regions should be kept separate from PCR reactions processing locations. Microcentrifuges and pipettes should be sterile which are used in the PCR laboratory. Suitable positive and negative controls has to be run every time while a sample is run.[104].

F) INHIBITORS AND ENHANCERS OF PCR

Inhibitors of PCR are:

- Heparin. Hence heparinized tubes should never be used for sample collection and only EDTA sample should be used. Blood collection in EDTA prevents coagulation.
- Porphyrin compounds found in blood, are also strong inhibitors of PCR. lysis of red blood cells and problems with white cell pellet formation occurs with Porphyrin. .

Other inhibitors include

- SDS,
- Proteinase K and
- Phenol, the commonly used reagents in nucleic acid extraction.

Enhancers of PCR include :

- Formamide,
- Dimethylsulfoxide,

- Polyethylene glycol,
- Glycerol, and
- DNA-binding proteins.

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR):

Single stranded RNA cannot be amplified by using PCR as it requires DNA template. However RNA can be amplified by producing cDNA from ssRNA with the help of enzyme reverse transcriptase. For this, dNTPs and an oligonucleotide primer which is complementary to 3' end of RNA to be amplified should be added. In presence of reverse transcriptase enzyme which act as a catalyzer in this reaction produces cDNA from the RNA template. This cDNA template which is produced in the above reaction act as template for first round of PCR. Though it has many applications it is mainly used for assessing the transcriptional activity of genes and gene isoforms.

Uses:

- mRNA expression can be quantified
- Differential gene expression can be analysed
- cDNAs can be cloned without producing cDNA library.

Though entire sequence are amplified in the standard PCR technique it is efficient in amplifying DNA fragment of < 5kb. DNA fragment which is more than 5kb containing both introns and exons are not suitable for PCR. Whereas in RT-PCR the resulting product is shorter as a result of splicing of the introns in the RNA and hence the end product cDNA. So instead of original DNA.

So PCR can be performed on the cDNA instead of the original DNA.

DETECTION OF FUSION GENES BY RT-PCR:

RT-PCR can be used to detect fusion genes as a result of chromosome translocation. Here both gene 1 and gene 2 are located on the same chromosome but at a distance greater than 20 kb which makes it impossible for PCR or southern blot hybridization to detect the translocation. To make detection possible the length of fragment has to be shortened by first transcribing it into RNA. Then the nuclear RNA is converted into mRNA by splicing and processing thereby removing the nucleotide bases present in between the gene 1 and gene 2. This mRNA forms the RNA template for RT PCR.

With the help of enzyme reverse transcriptase cDNA template is produced from mRNA. PCR is then performed on cDNA as template and

gene rearrangement produced as a result of chromosomal translocation is identified.[99]

RT-PCR of total RNA or mRNA can be performed by either one step or two step RT-PCR method.

One-step RT PCR - whole reaction from the production of cDNA to PCR amplification take place within a single tube.

The advantage of one step RT-PCR:

- Chances of contamination is less
- The procedure is simple as there is no need to transfer the cDNA synthesized into another tube for PCR
- Quick, sensitive and
- Results are reproducible.

Disadvantage :

Starting RNA template produced here prone to degrade, hence it is not used when repeated assays from the same sample is required.

Two-step RT PCR - Here the reverse transcriptase reaction and PCR amplification take place in two separate tubes.

Advantage:

- More accurate then one step RT PCR

Disadvantage:

- Contamination is more due to more frequent handling of sample[105]
- Promega is the Access RT-PCR system supplied by the manufacturer. Here the enzyme, reverse transcriptase and DNA polymerase used by the manufacturer are produced from Avian Myeloblastosis virus and thermos flavus.[104]

MOLECULAR MAKE-UP OF THE FUSION GENE:

The chromosomal breakpoint 9q34.1 corresponds to proto-oncogene c-*abl* which is the region corresponding to breakage in the t(9;22) translocation. C-*abl* gene represents the normal homologue of the transforming gene of *Abelson* murine leukemia virus, which causes B cell leukemia. The length of this gene is found to be 230kb, encoding at least 11 exons and its 5'end is oriented towards the centromere. Exons a1 and b1 are the two alternative first exons that are discovered.

Distance from Exon a1 to exon 2 is 19kb upstream, while distance from exon b1 to exon 2 is > 200kb upstream. Because of this genomic variation two types of mRNA transcripts are produced: one with 6-kb

mRNA coding from exons a1 to exon 2 with all its 11 exons, and 7-kb mRNA which codes from exon b1 to exon 2, skipping the 200-kb distance[106]. The protein product identified previously was p145 KD molecular weight. This protein corresponds to p145*abl.* This protein has weak tyrosine kinase activity. The protein is found to be associated with tyrosine phosphokinase enzymes thus indicating their involvement in normal cell growth control.

The breakpoint on chromosome 22q11.2 corresponds to breakpoint cluster region (*bcr*) gene on chromosome 22. The length of bcr gene is 130kb and trancribs into a 4.5 and 6.7-kb mRNA. The translation of this *bcr* gene with 4.5 and 6.7-kb mRNA produces 160-kD protein, p160*bcr.* This oligomerizes, autophosphorylates and transphosphorylates many other protein substrates. The increased tyrosine kinase activity of the bcr abl gene is due to the potentiating activity of the first exon sequences of the *bcr* gene which potentiates the tyrosine kinase of the *abl* gene when fusion of the gene occurs due to translocation. 89% of CML patients develop breakpoint within the first 175-kb of the c *abl* gene within the intronic region, between exons b1 and a1. *dbl* gene which is involved in the gate keeping of cell division beyond the S-phase of the cell cycle is homologous to *bcr gene,* particularly its central part involved in translocation.

The activity of GTP activating protein, p21, is present in the C-terminus of the *bcr. p21* belongs to the *ras* family of proteins that comes uder the family of GTP binding protiens[107]. Incontrast, some cases of CML develop *abl-bcr* fusion gene. This reciprocal hybrid gene is formed by interaction of p160*bcr* with the SH2 domain of abl gene. This hybrid gene is formed on chromosome 9q+ while the characteristic Philadelphia gene is formed on chromosome 22q-. Some CML cases have active transcription of this hybrid gene[108].

The Philadelphia chromosome formed by reciprocal translocation is a short chromosome 22, a result of t(9; 22)(q34; q11). This translocation produces a fusion BCR/ABL gene. mRNA that is formed has breakpoint in the BCR gene in the 5.8-kb major breakpoint cluster region called as M-bcr. In contrast, breakpoints on 9th chromosome occur in the *abl* gene. As a result of t(9,22), all exons distal to the point of breakage within M-bcr in 3' *bcr*-gene are completely removed and located again to chromosome 9. Exons in 5' *bcr*-gene comprising *bcr* exons 1 and 2, including exon 3 or without exon 3 remain on chromosome 22. The c-*abl* exons 2 (a2) through 11 are transposed to chromosome 22 and exons a1 and b1 may be added to it. During the mRNA formation process, the splicing acceptor site involved

in c-*abl* gene in exon a2 can skip splice donor sites in c-*abl* exons a1 and b1 to fuse with splice donor sites of the juxtaposed *bcr*. Two potential junctions are encoded depending on whether *bcr* exon 2 (b2) or *bcr* exon 3 (b3) is joined to c-*abl* a2. The fusion transcripts formed are designated as b2a2 or b3a2 depending on *bcr* exon b3, absence or presence. The presence of b2a2 indicate the non existence of *bcr* exon 3 due to the mechansim of alternative splicing. They differ in size and length by 75bp at the mRNA level and differ by 25 amino acids at the level of proteins. Both b2a2 and b3a2 have elevated tyrosine kinase activity, and both transcripts have same molecular weight of 210 kD (p210), and both of them can phosphorylate tyrosine residues on cellular proteins. The phosphorylation of tyrosine residues is similar to *v-abl* protein products phosphorylating activity.

The difference between p210 bcr-abl from the normal p145abl is that the end terminal of p210 bcr abl is substituted by *bcr* sequences. The p210 *bcr-abl* can transform hematopoietic stem cells *in vitro*[109] and this could be one of the many steps leading to leukemogenesis. The p210*bcr-abl* fusion protein has the ability of making the progenitors and granulocytes survive longer by inhibiting the genetically programmed normal determinants of cell death. This causes expansion of the cells in

the leukemic clone. Due to alternate splicing in some patients, coexpression of both transcripts b2a2 and b3a2 has been reported[110].

The breakpoints outside the M-bcr lead to various other rearrangements. The fusion protein of 210 KD encoded by BCR-ABL varies in kb, depending on breakpoint locations in the BCR gene between exons b2 and b3 or between exons b3 and b4 in a 3' or 5' gene rearrangement study. Depending on the breakpoint locations, two kinds of mRNA are created: b2a2 or b3a2. In most cases, cells have one of the two, b2a2 or b3a2 transcripts, but in 5-15% of cases alternative splicing results in the expression of two fusion transcripts too. The b3a2 transcript is larger by 75 bp.[111]

The fact that the p210 Bcr/Abl fusion protein can induce a CML-like disease provides strong evidence for their fundamental causal role in human CML[112]. Differences in the site of BCR breakpoint and the types of BCR/ABL transcripts suggest that Subtypes of CML can be separated, with different prognosis and clinical course. An increasing number of studies have shown no clinical impact of the site of breakpoint, M-BCR, or the type of BCR/ABL transcript.[113]

Three breakpoint cluster regions are identified till date: major (M-bcr), minor (m-bcr) and micro (mu-bcr). Maximum CML patients have a

p210BCR-ABL gene or Major-bcr, with mRNA transcripts being b3a2 and/or a b2a2 junction.

The BCR breakpoint site can be classified according to the presence of the rearranged band seen in the digested DNA after hybridization. In a study, 29% of the patients expressed the b2a2 transcript, whereas 62.3% expressed the b3a2 transcript. Both the b2a2 and b3a2 types of transcript were found in 8.2% of the patients in a study of 62 patients. The median duration of CP for patients with transcripts b2a2 and b3a2 was 39 and 42 months respectively. A statistically significant correlation between the two groups (b2a2 and b3a2) was observed in overall survival (p=0.0420). The patients with the b3a2 type of transcript survived significantly longer than the group expressing the b2a2 transcript. The longer duration of survival of the patients showing the b3a2 transcript expression may be caused by the less aggressive course of the accelerated or blastic phase. [114]

DETECTION OF GENE REARRANGEMENT IN CML:

The rearrangement of gene bcr-abl in CML cases which are ph+ leads to the production of mRNA transcripts of two different types, b2a2 and b3a2. Inorder to find the the transcripts, the RNA is first separated from the leukemic cells followed by reverse transcription to cDNA and

finally subjected to PCR. The amplified PCR products is then confirmed via Southern Blotting after which probing is done or yet another round of PCR (nested PCR) is done. Another method of verification is by DNA SEQUENCING. The methods involved in RNA extraction, the various requisites for PCR(eg. Temperature, enzymes, oligonucleotide primer, polymerase enzymes, etc) both of which are involved in the studies of bcr-abl gene rearrangement varies from lab to lab. [110] Inkeeping with the method illustrated by the manufacterur GibcoBrl, RNA is extracted from the white cells with the help of trizol LS reagent. RT-PCR is done in one step with the help of Promega Access system, NB1+ and Abl3 happen to be the first primer pair which is applied in the starting round of PCR. This lads to amplification of the genetic material between bcr exon b1 and abl exon a3 of the fusion bcr-abl gene. The resultant PCR products that have been amplified are subsequently introduced to the next round of PCR also called nested PCR with 35 cycles.

B2A and CA3 are the primer pair set which is unbound to the first set (nested primer) and are meant for detecting b2a2 and b3a2 transcript. CA3 and A2 aplifies the abl region which is not translocated. Hence this serves as the abl control. This test permits the detection of one ph+ cell amidst 103 normal cells. Subjection to nested PCR increases the detection rate to 1 ph+ amidst 106 normal cells.

Studies related to *Bcr-abl* gene rearrangement is of great use in making the diagnosis of CML. The drawback in cytogenetics is that the metaphase spread which is best for analysis is that may at times not be obtained. Yet another plus point over cytogenetics is that in conditions where bonemarrow is not available the test can be carried out with a peripheral blood sample for molecular analysis. This bcr-abl gene rearrangement studies can also be used to differentiate CML blast crisis from ALL. CML blast crisis will be ph+ bcr+, whereas ALL will be ph+ bcr-. This study will be of help in monitoring the course or progress of the disease. For eg. After BMT or treatment with alpha interferon , by detecting Ph chromosome we can assess the minimal residual disease.

MOLECULAR BASIS OF PH CHROMOSOME-POSITIVE ACUTE LEUKEMIA:

Point to be noted is that, in acute leukemia, Ph chromosome is linked not only with breakpoints within M – bcr(ph+ bcr+) rather this break can also be seen at 5' upstream of M-bcr. However it mostly comes within the first intron of bcr gene chromosome 22. This area is named the minor breakpoint cluster region (m-bcr).

Due to the rearrangement occurring within m-bcr, the fusion of bcr – abl gene leads to the formation of 7.0kb mRNA. This is attributed to the

fusion of the first exon (e1) of the gene bcr with the second exon a2 of the gene c-abl. The ultimate product of translocation is 190KD protein, bcr-abl with an increase in tyrosine kinase activity. It is found that p190 bcr-abl has an effect that is more potent than p210 bcr-abl.

Those leukemias bearing ph+bcr- rearrangements of m-bcr indicate de-novo acute leukemia. Such ph+ acute leukemia can be biphenotypic or a hybrid of myeloid-lymphoid lineage.

A) MYELOGENOUS

It has been reported tha the Ph chromosome is present in 1-2% of adult AML cases.when it comes to ph+ acute leukemia we encounter two varieties: one is CML presenting in myeloid blast crisis (Ph+ bcr+ AML) and the other is denovo AML(Ph+ bcr- AML)[52]

Facts that points to the diagnosis of CML presenting in myeloid blast crisis (Ph+ bcr+ AML) are as follows:

i) Blast crisis can manifest within days after diagnosis of Ph+ CML is diagnosed.
ii) Some cases showed features of additional cytogenetic abnormalities comparable to CML blast crisis.
iii) severe hepatosplenomegaly.

iv) Platelet counts are largely normal and there is intermittent increases of basophils.

v) certain signs and symptoms like weight loss, prolonged period of weakness.

vi) Poor prognosis like CML in myeloid blast crisis.

vii) when patient is in remission, the disease course appears like CML at chronic phase.

viii) Rearrangement at the M-bcr on chromosome 22, production of *bcr-abl* fusion gene and translation of p210 *bcr-abl* protein product, similar to CML resulting in the production of p210 *bcr-abl*.

Arguments in favour of de novo AML (Ph+ bcr- AML) are as follows:

i) Additional chromosomal abnormalities are different from those seen in CML myeloid blast crisis.

ii) The Ph chromosome is associated with breaks outside the M-bcr (at them-bcr) and a translation product of p190 *bcr-abl.*

B) LYMPHOCYTIC

Translocation t(9,22) is seen in 15-30% of adults with ALL and in 3-5% of childhood ALL. The presence of the t(9,22) in ALL carries a poor prognosis. Two groups of Ph+ ALL are reported by Molecular

studies. One is that CML itself presents in lymphoid blast crisis (Ph+ bcr+ ALL) and the other is *de novo* ALL (Ph+ bcr- ALL). Some adults and few children with ALL have rearrangement within the M-bcr and production of p210 (Ph+ bcr+ ALL or lymphoid blast crisis of CML). At remission, patients may revert to CML, and are Ph+. Rearrangement not within the Mbcr region is seen in some adults and virtually in all children with ALL, that is at the m-bcr(p190 *bcr-abl) with genotype* Ph+ bcr- ALL or de novo ALL. In CML with lymphoid crisis there occurs remission, with appearance of normal hemopoiesis.[52]

SOUTHERN BLOTTING:

A gene fusion that is specific for a particular translocation can be analysed by Southern blotting and PCR. In Southern blotting, restriction enzymes that are sequence-specific cut the DNA and fragments are separated by agarose gel electrophoresis based on the size variation of the fragment recognized by a specific probe for various fusions.

RISK ANALYSIS IN CML:

SOKAL SCORE:

The Sokal score[117] is derivative of 813 patients diagnosed with chronic phase of CML from a multivariate analysis of survival of patients between 1962 and 1981. Either single-agent chemotherapy or busulfan

treated cases are studied. % of blasts and spleen size were most strongly associated with survival. Formula and scoring (annexure 1).

HASFORD SCORE:

The Hasford score also known as Euro score[118] is a derivative from multivariate analysis of survival of early CML cases who were started on treatment between 1983 and 1994. Study included 981 patients with early CML. All cases studied were treated with only interferon alpha, or combination with another drug. The score was validated in a separate cohort of 322 patients. Formula and scoring:(annexure 2)

EUTOS RISK SCORE:

The European Treatment and Outcome Study risk score for CML was derived from response of 2060 patients by multivariate analysis, who were treated with imatinib between 2002 and 2006. The score is applicable to cases before starting therapy, at the time of diagnosis itself.[119] Formula and scoring (annexure 3).

AIMS AND OBJECTIVES

1. To correlate the following parameters: age, sex, clinical and haematological parameters of newly diagnosed CML patient to the disease course and cytogenetic findings.

2. To determine the frequency of various chromosomal abnormalities in CML cases at the disease diagnosis.

3. To determine the frequency of standard Philadelphia and variant translocation.

4. To quantitatively assess the nature of expression of the fusion product- b2a2 and b3a2 transcripts in Ph+ CML.

MATERIALS AND METHODS

A total of 30 cases were taken into study. All thirty cases were confirmed to be Chronic Myeloid Leukemia by blood counts, peripheral smear, bone marrow aspiration, cytogenetics and molecular genetics.

INCLUSION CRITERIA WERE:

i) Newly diagnosed CML cases not started on any chemotherapy.

Criteria for exclusion were:

i) any signs of accelerated disease, i.e. more than 10% myeloblasts and promyelocytes in the peripheral blood,

ii) prior treatment with any drugs such as busalfan, hydroxyurea, interferon, imatinib or nilotinib.

All 30 cases were evaluated with blood counts using a 5 part automated counter and verified manually with peripheral smear. Ultrasound abdomen and portal doppler was done at enrollment itself. The size of the spleen is assessed by real time b mode ultrasonogram performed in Institute of Radiology, Madras Medical College. Renal function test and liver fuction test was done in biochemistry lab. Coagulation profile (prothrombin time and activated partial thromboplastin time) was done in hematology laboratory. The bone

marrow aspirate was obtained from the patients and outsourced to Centre for Medical Genetics for cytogenetic analysis. Routine G-banding was done for karyotype analysis. Care was taken to see that bone marrow sample reaches the laboratory on the same day of collection. Peripheral blood from patients was sent for BCR-ABL qualitative analysis to SN GENE LAB,Surat.

Blood counts, serum chemistry, portal venous Doppler study, ultrasonogram of abdomen and coagulation profile were performed at enrollment, and blood counts monthly until the patient is on followup. The small size of the sample is due to economic constraints. Qualitative RT-PCR for the BCR-ABL transcript was routinely performed for determining the type of transcript and fusion product.

All 30 cases were started on imatinib after initial diagnostic workup and quantitative BCR – ABL was performed to assess the major molecular response (MMR) in selected cases only due to economic constraints.

MATERIALS FOR CYTOGENETIC STUDIES:

Cytogenetic study was performed in the Centre for Medical Genetics, Kilpauk, Chennai.

PRINCIPLE:

Bone marrow samples which contain spontaneously dividing cells are cultured short term without mitogen. Chromosome analysis of the dividing cells is useful in the diagnosis of malignancy and in the classification of hematological malignancies.

METHODOLOGY FOR CYTOGENETIC STUDIES:

A. SPECIMEN ACCESSIONING

Upon receipt in the laboratory, the sample is accessioned. Accessioning includes checking the patient's name, recording the volume and condition of the sample, and recording the time and date that the sample is received.

B. SPECIMEN REQUIREMENTS

1. ~2 mL of first or at least second tap marrow specimen freshly drawn into a sodium heparin vacutainer or syringe coated with heparin.
2. The marrow should have sufficient spicules.

C. REAGENTS AND MATERIALS (ANNEXURE IV)

D. SETUP

Bone marrow and leukemic blood specimens are setup according to the reason for referral and the age of the patient.

1. The specimens are setup as overnight (ON) / Two day (2d).

2. WBC's are to be checked in house for the following circumstances:
   a) The specimen received is from a pediatric patient.
   b) The specimen received looks very thick or thin. If the specimen is inadequate, setup an ON culture only
   c) The specimen received has been shipped form clients with great shipping distances or delayed arrival.
   d) The specimen received has a very high or low WBC indicated on the requisition form.

3. Use the WBC count to [10^6/mL] determine the volume of specimen needed to seed a culture.

4. Setup the following tubes and incubate at 37ºC with the caps loose. Label, T-25 culture flask with the patient's name, accession number, and the date received.
   a) 1 day tube / 2 day tube

Specimen depending on the count.

   b) Repeat patients

If there is adequate volume specimen, setup overnight culture.

c) If there is an inadequate specimen volume, setup at least one ON culture. Use your judgment for additional cultures.

d) If the WBC is higher or lower, use your own judgement.

F. HARVESTING

1) Add 40 µL of Colcemid to each 1 day/2 day culture early the next working day (~ 5:30 AM) and incubate at 37ºC for 1 hour.

2) Decant specimens in flasks to labeled centrifuge tubes.

3) Centrifuge tubes at 1100 rpm for 7 minutes.

4) Pour off the supernatant; gently resuspend the cells in 10 mL of freshly mixed hypotonic solution (8 mL of 0.56% KCL stock solution and 2 mL of distilled water) and incubate at 37ºC for 15-17 minutes.

5) Add 1 mL of fixative (3:1, Methanol to Acetic Acid); centrifuge at 1100 rpm for 7 minutes.

6) Pour off the supernatant; gently resuspend the cell pellet; add 10 mL of fresh fixative; let sit for 15 minutes.

7) Pour off the supernatant; gently resuspend the pellet in fresh fixative; centrifuge for 7 minutes.

8) Pour off the supernatant; gently resuspend the pellet in a quantity of fix to make a reasonable cell suspension for preparing slides

9) Drop the cell suspension on a microscope slide; evaluate on a phase microscope; make two more slides.

10) Bake slides at 90ºC for 1 hour. Follow the routine banding procedure.

G.ANALYSIS

1) Analyze 20 metaphases for each specimen. If a sufficient number of metaphases are not available, analyze as many as possible. Analysis includes, identifying members of each group, checking for all bands in both homologues, identifying sex chromosomes and counting total chromosome number.

2) Select three metaphases for karyogramming if all metaphases are identified as normal.

3) If the metaphases are not analyzable because of several complex abnormalities and / or several unidentifiable markers, count the total chromosomes and select metaphases to be imaged for review.

4) If the case is abnormal, select two metaphases from each abnormal clone for karyogramming.

MOLECULAR ANALYSIS:

All samples were tested in SN gene lab Surat, India. Whole buffy-coat cells were used. RNA extraction, RT-PCR, and real-time quantitative PCR were performed according to WHO recommendations. Real-time quantitative PCR was performed on an ABI PRISM 7700 sequence detector (Perkin Elmer). ABL was used as housekeeping gene to correct differences in RNA quality and/or RT efficacy. BCR-ABL and ABL plasmid dilutions (Ipsogen) were used as standards. The final results were calculated as the ratios of BCR-ABL to ABL and are expressed as percentages. All experiments were performed in duplicate, and the results are expressed as the percent ratio to ABL. The BCR-ABL:ABL ratios were further multiplied by the conversion factor of the Bologna laboratory to set the results on an international scale. Qualitative BCR-ABL was assessed by similar RT-PCR. RNA extracted, using many primers, specific for different transcripts of various sizes, were amplified by PCR. This PCR product is made to run on agarose gel and electrophoresed against gel matrix containing known probes of various BCR-ABL fusion transcripts. All samples were tested for following transcrips: b2a2 (p210), b3a2 (p210).

METHODOLOGY FOR MOLECULAR STUDIES:

Reagents and consumables used in PCR must be sterile. For molecular studies autoclaving of all test tubes, pipette tips and containers should be done to prevent contamination. Wearing gloves and necessary sterile precautions should be taken. RNA extraction and reagents disposal should be done with a new pair of gloves. RNA extraction and PCR amplification steps should be performed in separate rooms.

EXTRACTION OF WHITE CELLS:

a) 1 ml of whole blood is diluted with 0.9% saline of about 1 ml.

b) diluted blood should be layered on top of ficoll-paque of 1ml in a 15 ml conical centrifuge tube which is sterile.

c) a refrigerated centrifuge is used to centrifuge at 4^0 c for 25 minutes at a rate of 2500 rpm.

d) white cell layer should be aspirated into a 15 ml sterile centrifuge tube with 0.9% saline of about 10ml.

e) a refrigerated centrifuge is used to centrifuge at 4^0 c for 25 minutes at a rate of 1500 rpm.

f) supernatent should be discarded and cells are resuspened in 10 ml of 0.9% saline

g) a refrigerated centrifuge is used to centrifuge at 4^0 c for 25 minutes at a rate of 1500 rpm.

h) supernatent should be discarded and cells are resuspended in 10 ml of 0.9% saline

   a. Aliquot of 1 ml each should be added into cryovial and store frozen at -70^0 C

RNA EXTRACTION

a) frozen white cells of amount of one cryovial should be thawed to room temperature.

b) Into an ependorf tube with a capacity of 1.5ml, containing 750 ul trizol, 250 ul of the white cells are added.

c) allow to stand for 5 minutes

d) 200 ul of separating reagent is added to the BCP phase.

e) This mixture should be shaked vigorously for 40 seconds and allowed to stand for 15 minutes.

f) this mixture is again centrifuged at 13,600 rpm in a microfuge for 15 minutes.

g) after centrifugation the mixture is separated into 2 phases, a pink coloured lower phase and aqueous upper phase.

h) Into an ependorf tube with a capacity of 1.5ml, containing 500 ul isopropanol, the upper phase is added.

i) allow the mixture to stand for 10 minutes after mixing it.

j) again the mixture is centrifuged at 13,600 rpm for 10 minutes

k) discard the supernatent and the RNA pellet is resuspend in 1000 ul of 70% ethanol

l) now the mixture is again centrifuged for 10 minutes at 13,600 rpm.

m) discard the supernatent and dry the RNA. But the RNA should not be dried completely as this would make the RNA difficult to dissolve.

n) RNA is dissolved in 50ul – 200ul RNAse DNAse free water.

o) RNA is heated for 10-15 minutes in a water bath at 55-60^{0}C.

p) this RNA is stored at –70^0 C.

ONE STEP RT-PCR:

a) name a tube as M and thaw it.

b) to tube M, 0.5 ul *AMV* and 0.5 ul *Tfl* is added.

c) to this tube M 2.5 ul RNA is added.

d) this tube M is incubated in a thermal cycler for:

i. at 48^0C for 46 minutes and this is followed by 2 minutes incubation at 94^0C.

ii. following which 35 cycles are run with each cycle consisting of 3 set of temperatures: at 64^0 C for 1 min, 74^0 c for min.

iii. a final extension step, 73^0 C for 10 minutes

e) A control tube M with *AMV* and *Tfl* but without RNA is incubated in the above reaction in the thermal cycler.

NESTED PCR:

a) Take two tubes A and B and thaw them.

b) 1 ul of material is taken from the PCR product of each tube M and added to tubes A and B.

c) both tubes A and B are incubated in a thermal cycler at temperature of:

i. 96^0 C for 2 min

ii. followed by 35 cycles, with each of the cycle consisting of 3 temperatures of 96^0c for 1 min, at 64^0 C for 1 min, 72^0 C for 1 min and

iii. finally an extension step of 73^0 C for 10 minutes

d) these nested PCR products in tubes A and B are stored at -70^0C if they are to be electrophoresed later.

AGAROSE GEL ELECTROPHORESIS

a) 2% agarose gel is prepared by dissolving agarose of 0.8g in 40 ml in TBE buffer in a microwave oven.

b) ethidium bromide of 1ul is added to (a).

c) the agarose gel that is melted is poured into a tray with a comb to make the sampe wells (Easy-Cast)

d) after the solidification of agarose occurs, the comb is removed and the gel in a refrigerated at 4^0 C for further use. The gel should not be kept for more than a day.

e) 1 ul of the nested PCR product together with 1 ul of loading dye and 6 ul of distilled water is put on a piece of paraffin. This is mixed and loaded into a well on the agarose gel card.

f) the molecular marker containing mixtures of 1 ul of 100bp ladder along with 10 ul of distilled water and 2 ul of loading dye on a piece of paraffin is mixed and 6 ul is loaded at the opposite ends of the wells.

g) the electrophoresis is run at 130V for an hour or until the dye reaches three-quarter of its way over the gel.

Photography

a) the gel is now viewed under UV light using a transilluminator.

b) the gel is photographed using the camera system.

c) Interpretation of results is based on the position of the bands from the wells containing samples in tubes A and B, with the 100 bp ladder as a molecular marker.

OBSERVATION AND RESULTS

A total of 157 leukemic patients attended outpatient Department of Hematology from january 2013 to august 2014. Of 157 cases, 48 cases were diagnosed Chronic Myeloid Leukemia. Of 48 CML patients, 30 patients of CML were taken into study. 8 cases were lost for follow up. In 10 cases cytogenitics at diagnosis was not done.

TABLE 1: Parameters of 30 CML cases involved in study

S.No	AGE	SEX	KARYOTYPE
1	45	F	46xx,t(9,22)(q34;q11.2)
2	44	M	46xy,t(9,22)(q34;q11.2)
3	45	M	46xy,t(9,22)(q34;q11.2)[3]/48,idem,+8,inv(12),+21[22]
4	39	M	46xy,t(9,22)(q34;q11.2)
5	43	M	46xy,t(9,22)(q34;q11.2)
6	65	F	46xx,t(9,22)(q34;q11.2)
7	49	M	46xy,t(9,22)(q34;q11.2)
8	45	F	46xx,t(9,22)(q34;q11.2)
9	46	M	46xy,t(9,22)[8]/49xy,idem,+8,+19,t(9,22)[18]
10	24	F	46xx
11	24	M	46xy,t(9,22)(q34;q11.2)
12	36	M	46xy,t(9,22)(q34;q11.2)
13	49	M	46xy,t(9,22)[14]/ 47, idem, +der(22) t(9,22)[22]
14	26	M	46xy,t(9,22)(q34;q11.2)
15	52	M	46xy,t(9,22)(q34;q11.2)
16	60	M	46xy,t(9,22)(q34;q11.2)
17	60	F	46xx,t(9,22)(q34;q11.2)
18	45	m	46xy,t(3,6,9,22)(p13;p21;q34;q11.2)[25]
19	35	m	46xy,t(9,22)(q34;q11.2)
20	24	M	46xy,(9,22)(q34;q11.2)
21	41	M	46xy,(9,22)(q34;q11.2)
22	24	M	46xy,(9,22)(q34;q11.2)
23	24	M	46xy,t(9,22)(q34;q11.2)[3]/45x, idem, -y[22]
24	57	F	46xx,t(9,22)(q34;q11.2)
25	46	F	46xx,t(9,22)(q34;q11.2)
26	43	M	46xx,t(9,22)(q34;q11.2)
27	30	M	46xy,t(9,22)(q34;q11.2)
28	29	M	46xy,t(9,22)(q34;q11.2)
29	40	F	46xx,-1,der(6)t(1,6)t(6,13),der(7)t(1,7),t(9,22),del(10)+mar[23]
30	29	f	46xx,t(9,22)(q34;q11.2)

30 cases of newly diagnosed CML, not started on any treatment was included into study. Non-modifiable risk factors, clinical parameters, karyotypes observed were compared with each other. Risk analysis was done using Sokal, Hasford and EUTOS scoring systems.

23 cases had only t(9,22)(q34,11.2), 1 case had variant translocation, 5 cases had major and minor additional chromosomal abnormality and case had normal karyogram inspite of BCR-ABL gene being positive. 27 of 30 cases showed only abberant clone. Only 3 cases showed normal metaphases (46xx and 46xy) in parallel to aberrant clone. In 3 of 4 patients with major route ACA, complex aberrant karyotypes with 3 or more chromosomal alterations were detected.

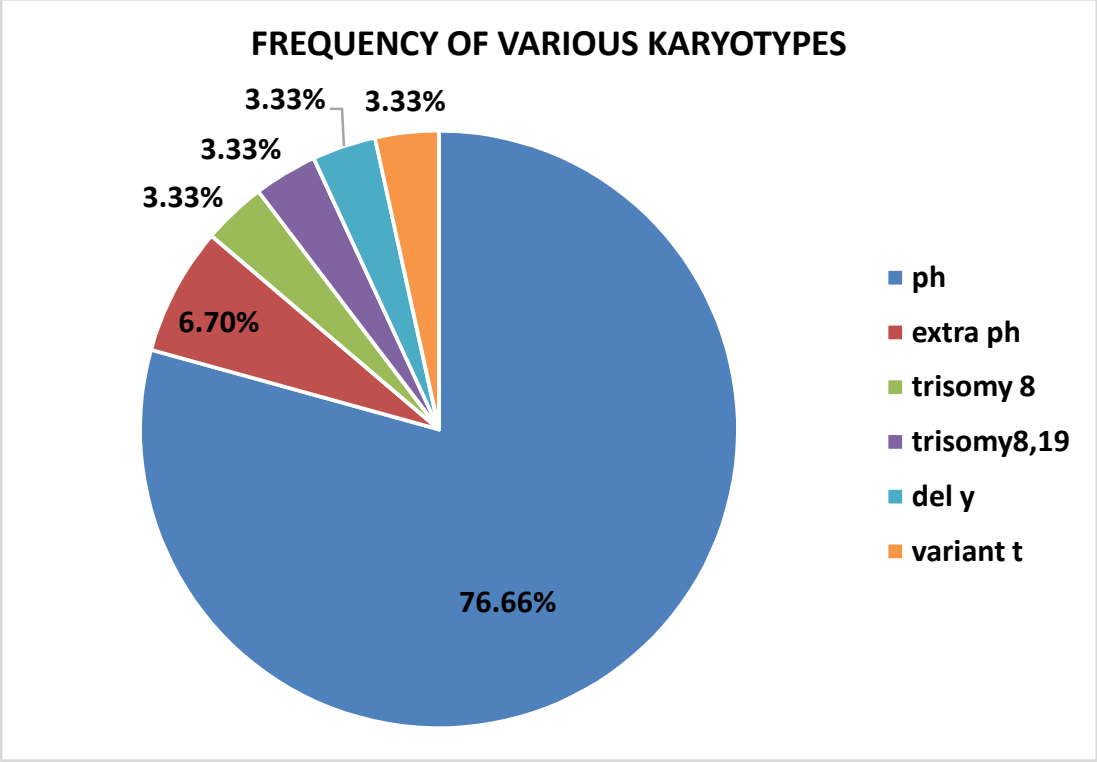
Frequency of expression of various karyotypes:

76.66% (23 cases) presented with only Philadelphia translocation t(9,22). 20% (6 cases) had additional chromosomal abnormalities in addition to Philadelphia chromosome. These ACA observed were 6.7% (2 cases) with extra Philadelphia chromosome, 3.33%(1 case) with trisomy 8, 3.33%(1 case) with trisomy 8 and trisomy 19, 3.33%(1 case) with –y ond 3.33% with other rare abnormalities like der(6), t(1,6), t(6,13), der(7), t(1,7), del(10), +mar, 3.33% (only 1 case) had complex

variant translocation involving chromosomes 3, 6, 9 and 12 and 3.33% (1 case have normal karyotype without expressing t(9,22)) but BCR-ABL+ and case is included under cases with t(9,22) itself for statistical purpose. To confirm the karyotypic abnormality FISH was planned but withdrawn due to financial constraints.

Table 2 : Frequency of expression of various Karyotypes

Karyotypes	**frequency**	**%**
Ph only	23	76.66%
Ph -, BCR – ABL +	1	3.33%
Additional chromosomes	5	16.67%
• extra ph	2	6.7%
• + 8	1	3.33%
• + 8, + 19	1	3.33%
• - Y	1	3.33%
• Variant translocation	1	3.33%



Majority of the cases have t(9,22) involving locus 34, 11.2 of long arm. Among the additional chromosomal abnormality extra Philadelphia chromosome and trisomy 8 was the most common abnormality observed. Among trisomy 8, one patient had additional trisomy 19. Other abnormalities have equal incidence. Frequency of various karyotype expression is shown in table 2.

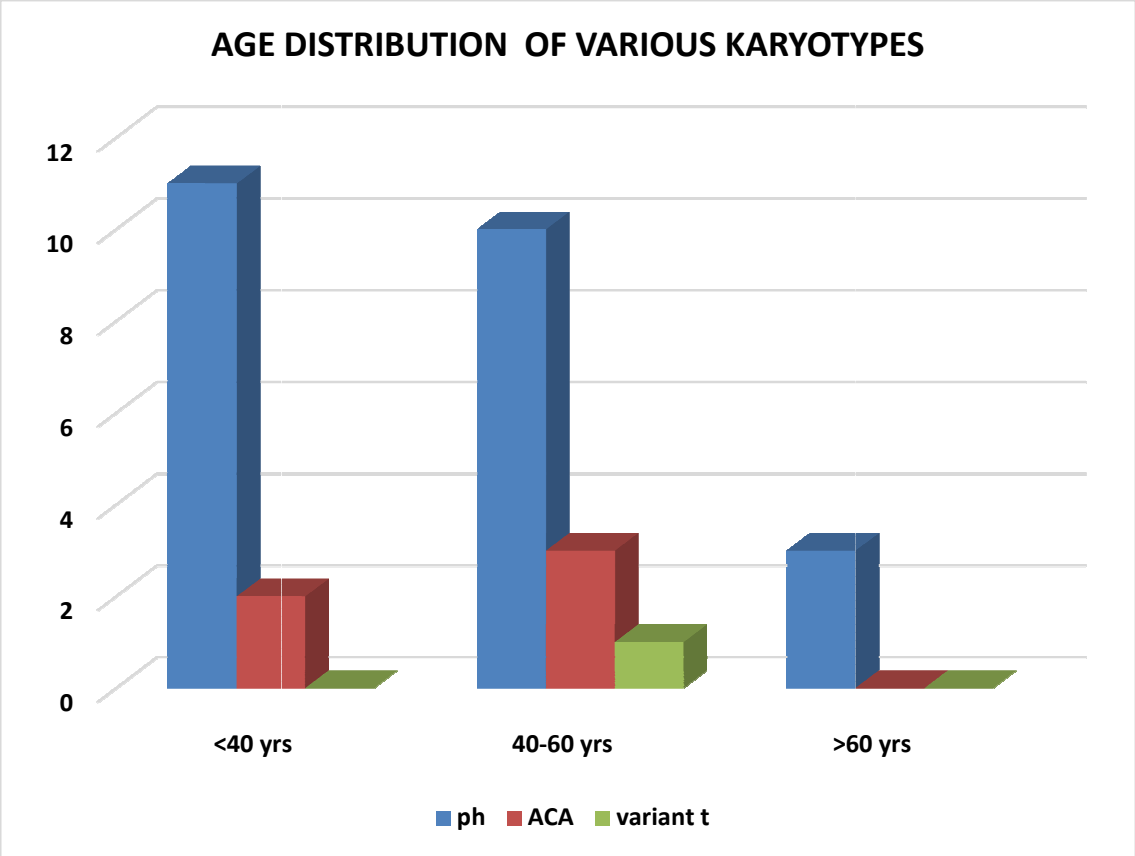
Age distribution:

Majority of the cases, 46.66% (14 cases) presented in the age group of 40 – 60 years. 43.33% (13 cases) presented in the age group of less than 40 years and only 10%(3 cases) presented in >60 years of age. Table 4 shows age distribution of cases. Thus majority presented in the

age group of 40-60 years. Among 46.6% in the age group of 40-60 years, 71.42% have translocation 9,22 only and 21.42% have additional chromosomal abnormalities. In <40 years age group, 84.16% have t(9,22) and only 15.38% have additional chromosomal abnormalities. Thus additional chromosomal abnormalities were observed maximally in the age group of 40-60 years only. Since very few number of cases were registered >60 years, significant correlation of age with chromosomal abnormality cannot be arrived at. Although no ACA were detected above 60 years, ACA above 60 years cannot be excluded altogether.

Table 3 : Age distribution of various karyotypic abnormality

Age range	**Ph+**	**Ph+addi chr.abn.**	**Variant t**	**% total in each age group**
< 40 Years	11 (84.61%)	2 (15.38%)	0%	43.33%
40 – 60 Years	10 (71.42%)	3 (21.42%)	1(7.14%)	46.66%
> 60 Years	3(100%)	0%	0%	10.00%

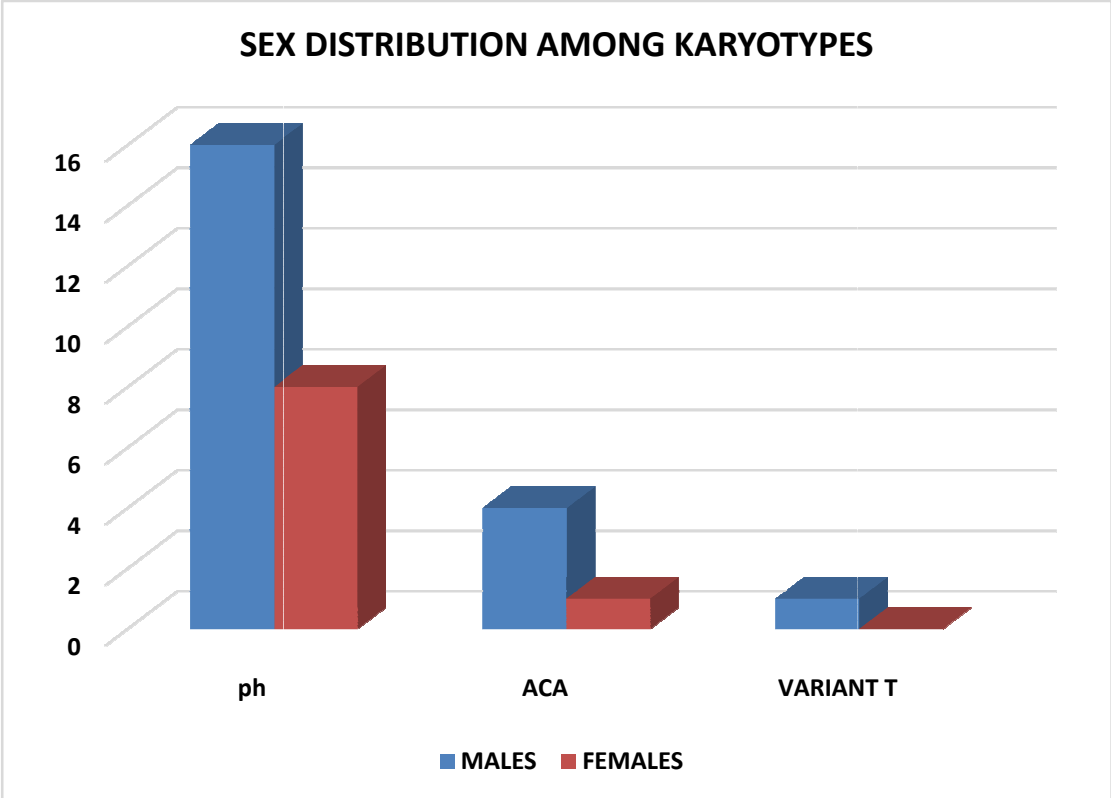


Sex distribution:

A total of 21 males and 9 females were analysed randomly. Table 5 shows sex distribution among various karyotypes. On evaluating the sex distribution of karyotypic abnormalities, 70% (21 cases) were males and 30% (9 cases) were females. Hence, disease was found to be associated more commonly among males than females.

Table 4 : Sex distribution among various karyotypes

sex	Ph+	Ph+ addi chr.abn.	Variant t	Total % of each sexes
Males	16 (69.56%)	4 (80%)	1 (100%)	21 (70%)
Females	8 (33.33%)	1 (20%)	0	9 (30%)



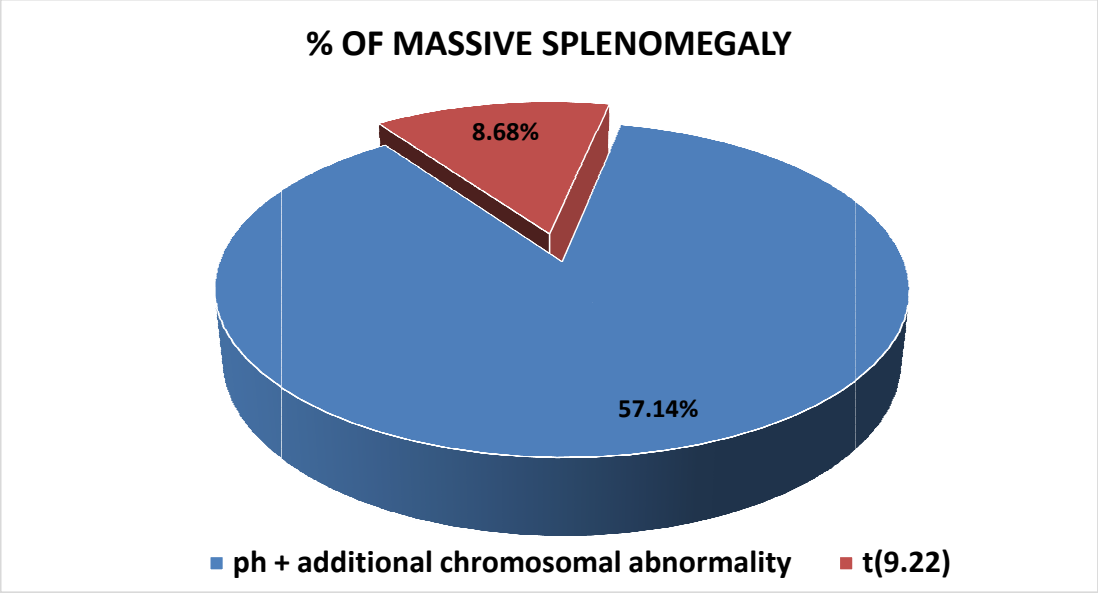
Among 76.66% with Philadelphia translocation 69.56% were males and 33.33% were females. And among 20% with additional chromosomal abnormalities, 80% were males and 20% were females. In view of single case with variant translocation, it is not possible to consider the value as statistically significant.

Spleen size and karyotype abnormalities:

All 30 cases had splenomegaly ranging from moderate to massive degree. None of them had mild splenomegaly. 76.66% (23 cases) had moderate splenomegaly between 11-20cm and 23.33% (7 cases) had massive splenomegaly of >20cm. table 5 shows the degree of splenomegaly and karyotypic abnormality.

Table 5: Data showing severity of spleen size with karyotype frequency

Spleen size	Ph+	Ph+addi chr.abn.	Variant t	Total
11-20 cm	21 (91.30%)	1 (4.34%)	1 (4.34%)	23
>20 cm	3 (42.86%)	4 (57.14%)	-	7



Comparing the spleen size between those with Philadelphia only and those with additional chromosomal abnormalities, 57.14% of those with additional chromosomal abnormalities had massive splenomegaly compared to 42.86% of them with Philadelphia and massive splenomegaly.

Leucocytosis and chromosomal abnormality:

Taking total leucocyte count of more than 200,000 into consideration 3 patients (75%) out of 4 with ACA have leucocytosis of >2lakhs when compared to 15 patients (57.69%) out of 44 with non-ACA. Hence the presence of leucocytosis of more than 200000/dl is associated with poor prognosis.

Table 6 : Comparison of TLC among AC and non-ACA

TLC	> 200,000/dl	< 200,000/dl
ACA	3 (75%)	1 (25%)
NON-ACA	15 (57.69%)	11 (42.31%)

Platelet Count and Abnormal Karyotypes:

Generally CML is characterised by thrombocytosis. Abnormally high platelet counts of more than 600,000/dl is found in 50% of patients with ACA while only 15.38% of patients with non-ACA have thrombocytosis. So thrombocytosis of more than 600,000/dl is associated with adverse outcome.

Also only one patient had thrombocytopenia and that to with abnormal ACA with two cell lines, one cell line with standard Philadelphia and other cell line with Philadelphia, trisomy 8, inversion 12 and trisomy 21 in 22 metaphases.

Table 7: Comparison of platelet count among ACA and non-ACA

Platelet count	> 600,000/dl	< 600,000/dl
ACA	2 (50%)	2 (50%)
NON-ACA	4 (15.38%)	22 (74.21%)

PT and aPTT in CML:

3 of 30 cases had abnormally prolonged prothrombin time of >20 sec and 16 of 30 cases had abnormally prolonged prothrombin time of >38sec. This abnormal prolongation can be attributed to hepatic compromise and/or acquired inhibitors and further studies have to be done to find out the cause. There was no significant prolongation in cases with ACA as compared with standard Philadelphia translocation.

Table 8 : Characteristics of patients with major route ACA

No.acc. to table1	Karyotypes (short) in addition to t(9,22)	% of metaphases with ACA	Follow up period (mon)	Course
3	+8, inv(12), +21	88%	4	Thrombocytopenia, renal compromise necessitating dialysis
9	+8, +19, extra ph	72%	18	Unexplained leucopenia and thrombocytopenia warranting stoppage of imatinib
13	+der22 extra ph	88%	18	No progress in disease. On nilotinib from day1
29	-1, der(6)t(1,6) t(6,13), der(7)t(1,7), del 10, + mar	92%	18	Acceleration after 1 year so dose of imatinib is increased. Develops syptoms pertaining to mastocytosis

Risk Categorization of Cases:

Hasford and Sokal index scoring systems are available to predict survival duration of CML patients on imatinib therapy.

Sokal Scoring System:

Sokal score is calculated by taking into account the following parameters: age of the patient, spleen size, platelet count and blast% in peripheral blood. Table 6 shows the sokal scoring for all cases according to the karyotype expressed.

Table 9 : Sokal Score

Karyotype	Low	Intermediate	High
Ph only	1 (4.30%)	7 (30.43%)	16 (66.66%)
Ph+ additional chromosomal abnomality	-	2 (33.33%)	4 (66.66%)

According to the sokal scoring, 65.21% (15 cases) with Philadelphia translocation and 66.66% (4 cases) with additional chromosome abnormality were found to have high risk.

Hasford scoring :

Hasford score is evaluated with following parameters: age, spleen size, platelet count, peripheral blood blast%, eosinophil% and basophil%. Table 7 shows hasford score between Philadelphia and abnormal karyotypes.

Table 10 : Hasford Score

Karyotype	Low	Intermediate	High
Ph only	-	19 (79.16%)	5 (21.73%)
Ph+ additional chromosomal abnormality	-	5 (80.00%)	1 (20.00%)

Hasford scoring shows 80% of those with additional chromosomal abnormality and 78.26% with Philadelphia translocation was found to have intermediate risk. 21.73% of cases with Philadelphia translocation have high risk and 20%with additional chromosome abnormality have high risk.

EUTOS scoring:

Parameters taken into scoring are: Basophil% and spleen size.

Table 11 : EUTOS Score

Karyotype	Low	Intermediate
Ph	5 (21.73%)	19 (79.16%)
Ph+ Additional Chromosomal Abnormality	-	6 (100.00%)

Eutos scoring shows 100% high risk for those cases with additional chromosomal abnormality and only 78.26% of those with Philadelphia have high risk.

Table 12 : Comparison of Hasford and Sokal scoring system

Score	Sokal	Hasford
Low	1	0
Intermediate	9	24
High	20	6

Table 13 : Comparison of scoring system in cases with ACA

Karyotype	Category	Sokal	Hasford	EUTOS
PH+additional chr abn.	intermediate	2	5	0
	high	4	1	6

In 6 cases with Philadelphia translocation with additional chromosomal abnormality,2 cases were classified as intermediate risk and 4 cases were classified as high risk by Sokal, 5 cases as intermediate risk and 1 case with high risk by Hasford and all 6 cases as high risk by EUTOS.

Thus risk scoring was found to have no significance in this study.

DISCUSSION

CML accounts for 20% of all types of leukemia. 30 cases were included into the study and completely investigated with complete blood counts, cytogenetic analysis and bcr-abl qualitative assay.

The incidence of Ph+ CML in patients was found to be 76.6%, compared to literature, which reports 85% - 95% (Cartwright et al,1992). According to Block et al,1999, 10 – 20% of Ph+ CML patients have ACA at diagnosis. In the current study 13.33% of Ph+ CML patients have ACA.

Redaelli, Bell, Casagrande et al, stated the common age group of presentation of CML to be 40 – 60 years. Our study also confirms the common age group affected to be 40 – 60 years.

Among sexes, males are predominantly affected than females, according to Wintrobe, Hasenbush 1939 and David G Savage et al,1996, which reconciles with our study.

57.14% with ACA have massive splenomegaly compared to 42.86% of Ph+ CML with massive splenomegaly.

The most significant observation we found in the study was the negative impact of major route ACA from the time of diagnosis to the duration of progress free survival (PFS) and MMR. In patients with ACA, though the observation period was kept to a minimum of 36 months, it showed a worse outcome which was significant and meaningful, inspite of the fact that only a small number of 5 patients had ACA.

According to Fabarius et al, all other additional cytogenetic findings at diagnosis (ie, variant translocation, minor ACA) had no recognizable impact on prognosis. In our study, 1 case with complex variant translocation had no progress in disease course and the patient is doing well with nilotinib from the start.

According to Haferlach and Fabarius et al 2011, trisomy 8 was the most common chromosomal abnormality presenting in combination with various chromosomal abnormalities, followed next by isochromosome (17)(q) and extra Ph chromosome.

Our study also showed the most common additional chromosomal aberrations presenting at the time of diagnosis to be trisomy 8 and extra Ph.

The study also states that median age of patients with ACA was lower than that of the other groups. But in our study the median age for patients with ACA was 38.5%.

According to Cortes, Talpaz et al, only major route ACA had prognostic impact, not minor route ACA. Major route ACA presenting as complex cytogenetic aberrations involving three or more chromosomes at the time of diagnosis showed poor prognosis. The study also shows that 5 of 6 patients had progression of disease from chronic to accelerated phase, thereby decreasing PFS period. On the other hand, patients with minor route ACA had no significant impact on PFS.

In our study of 4 patients with major route ACA, all 4 have complex cytogenetic aberrations involving 3 or more chromosomes with median age of presentation being 43.33% and most commonly affecting males. In accordance with literature, most common abnormality was found to be trisomy 8 and extra Philadelphia.

In patients with ACA, it was found that leucocytosis of more than $200x10^9$/l in 75% and thrombocytosis of more than $600x10^9$/l in 50% as against 57.69% and 15.38% in non-ACA was recorded which goes handinhand with literature by David G Savage et al,1996 who also shows

patients with ACA are likely to present with greater degrees of leucocytosis and anaemia.

Rowe et al, 1983 and Mason et al,1974 showed that patients with additional chromosomal aberrations have greater degrees of thrombocytosis when compared to patients with standard Philadelphia translocation.

On assessing the disease course in patients with chromosomal abnormality other than standard Philadelphia translocation, all additional cytogenetic abnormality at diagnosis including variant translocation, -y , but not major route ACA, had no impact on prognosis. Fabarius et al,2011 also derived at similar results. In our study, 1 case with complex variant translocation had no progress in disease course and the patient is doing well with imatinib from the start. Also our patient with –y showed similar disease course as patients who harbour standard t(9,22) according to the report of Ei-Zimaty et al 2004 and Marzocchi et al 2011.

Merlin et al,1996 found that the percentage of metaphases involved by ACA has dependence on nature of cytogenetic aberration. But this was disproved by Fabarius et al,2011 who reported that 59% of median metaphases are affected by major-route ACA, while only 85% of metaphases are affected by minor-route ACA. Hence the impact produced

by cytogenetic aberrations on the disease course is independent of the percentage of metaphases affected. In current study the median percentage of metaphases affected in major route ACA was found to be 85% and median metaphases affected in minor ACA was found to be 94% but these 94% affected metaphases have no bearing on prognosis thus proving the reports of Fabarius et al that impact of cytogenetic aberrations depends on the type of ACA independently of the percentage of metaphases affected.

In the past since all cytogenetic aberrations including major-route ACA, variant translocation, -Y, other minor-route ACA are evaluated as a whole, no prognostic impact can be detected.

We analysed the duration of PFS comparing between groups with only t(9;22), variant t, -Y, and major and minor ACA. After an average observation period of 13 months, the median times to PFS was 12 months for t(9,22), 12 months for t(v,22) and 10 months for –Y. This shows that there was no significant difference in the duration of PFS among those with minor ACA, whereas the duration of PFS was further reduced to 6 months in cases with major ACA. Although the sample size is too small and follow up period is short, PFS was significantly reduced in patients with major ACA which cannot be deglected altogether. Fabarius et al

reported, the 5-year PFS of standard t(9;22) was 90%, for t(v;22) it was 81%,for –Y it was 88%, for minor-route ACA as 96% and for major-route ACA as 50% (*P* <.001) and the 5-year overall survival was 92%, 87%, 91%, 96%, and 53% with *P vaiue* < .001 respectively. Thus he concluded that Variant t and deletion Y had no significant influence on PFS or OS.

Comparing the survival and disease course between patients with ACA and non ACA, 24 of the 24 patients with t(9,22) and 2 of 2 patients with minor-route ACA were still alive, whereas 1 of 4 patients with major-route ACA, who had trisomy 8 in addition to trisomy 19 and extra Ph had persistent pancytopenia warranting stoppage of imatinib and he developed severe leucopenia and scummbed to sepsis. Other patient with trisomy 8 in addition to trisomy 21 and inversion 12 had severe renal compromise warranting dialysis. The other patient with translocation involving chromosome 1, 6, 7 and deletion 1, 10 went in for acceleration after a period of 6 months of treatment with imatibib who attained remission and now doing better with increased dosage of imatinib. And the patient with derivative 22 along with t(9,22) is doing better with nilotinib from the start. Thus major route ACA were found to have poor prognosis and as reported by Kantarjjian et al, the chromosomal aberrations are non-random.

The feature of nonrandom association of chromosomal aberrations associated with survival and disease progression is seen in other cancers which is considered as the principle probably for maintanence of carcinogenesis. (Fabarius and Willer et al 2002). Although an adverse progression is associated with more than one chromosomal abnormality. Second Ph chromosome, isochromosome(17)(q10) and trisomy 8 which has unfavourable prognosis.

According to literature patients with CML are more prone to develop systemic mastocytosis and patients with systemic mastocytosis have trisomy 8, trisomy 9 and deletions of chromosome 1,7,9 and 11. Our patient with ACA had mean basophil count of 30 with symptoms of severe uncontrolled itching and mastocytosis with one patient harbouring trisomy 8 and other patient had abnormalities involving chromosome 1 and 7, suggesting that these patients are more susceptible to evolve into systemic mastocytosis and finally basophilic leukemia for which monitoring should be done appropriately.

At diagnosis the patients with additional cytogenetic findings is 20% and that of variant translocations is 3.33%, ACA is 9.7% and -Y is 3.3% which remains within the range of published data.

At diagnosis, though the patients with major route ACA is small at about 13.33%, but still its impact on prognosis is evident.

We conclude that the analysis of presence of ACA at diagnosis is very essential in CML since the presentation is heterogeneous. Compared with other patients, those with major route ACA had poor prognosis and these cases require close monitoring and early intervention by stem cell transplant.

SUMMARY

CML accounts for 20% of all leukemias. >90% of patients present in chronic phase although 95% of those presenting in chronic phase of CML have reciprocal translocation involving chromosomes and 22, the remaining 5-6% cases show additional chromosomal abnormalities at the time of diagnosis. These ACA were divided into major ACA, minor ACA and variant translocations. The significance of cytogenetic analysis at the time of diagnosis in patients with CML is the negative prognostic impact that they impart on the disease course and survival.

In this present study of 30 cases of CML, 6 of them had abnormal cytogenetics in addition to standard Philadelphia translocation.of the 6 cases, 4 had major ACA while 2 had variant translocation and deletion Y. cases with major ACA were found to have leucocytosis of >2 lakhs, thrombocytosis of >6 lakhs and mean basophil count of 30 in the bone marrow sampling with the most frequent cytogenetic abnormality behind trisomy 8 and extra Philadelphia chromosome.

The duration of progress free survival in patients with major route ACA was found to be reduced significantly to a duration of 4 months as against patients with minor ACA and t(9,22), where duration of progress

free survival is very much prolonged with an average of 15 months and none of the patients went into acceleration in the entire observation period.

Thus we conclude that cytogenetic analysis at the time of diagnosis carries prognostic significance to the disease and hence it should be carried out on all cases irrespective of the phase of presentation. Also the presence of major ACA has poor prognosis.

Though the role of bone marrow biopsy and bcr abl gene transcripts are not included in the study, further studies may help in analysing their significance.

CONCLUSION

- All cases of CML should be evaluated with cytogenetics as a part of initial diagnostic workup itself.

- This helps in categorising the patients into various risk groups based on the presence or absence of Additional Chromosomal Abnormality (ACA) and knowing the type of ACA, thereby deciding the mode of treatment to be given and the nature of care to be delivered to these patients.

- Major Additional Chromosomal Abnormality (ACA) has poor prognosis and most common ACA is trisomy 8 and Extra Philadelphia.

- The duration of progress free survival in patients with major route ACA was found to be reduced significantly to a duration of 4 months as against other patients.

- Depending on the type of cytogenetic abnormality and the prognostic impact of abnormality, these cases require close monitoring and early intervention by stem cell transplant.

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ANNEXURE 1

SOKAL SCORING:

The score, which is a hazard ratio, is calculated using the following formula:

exp (0.0116 x (age [years] – 43.4)) + (0.0345 x (spleen size [cm] – 7.51) + (0.188 x ((platelets [10^9/L]/700)^2 – 0.563)) + (0.0887 x (blasts [%] – 2.10)).

Sokal et al proposed three risk groups:

Low-Risk **(score < 0.8, 39% of patients),**

Intermediate-Risk **(score 0.8 - 1.2, 38% of patients) and**

High-Risk **(score> 1.2, 23% of patients).**

ANNEXURE 2

HASFORD SCORING:

The score is calculated using the following formula:

(0.6666 x age [0 when age < 50 years; 1 otherwise]) + (0.0420 x spleen size [cm]) + (0.0584 x blasts [%]) + (0.0413 x eosinophils [%]) + (0.2039 x basophils [0 when basophils < 3%; 1 otherwise]) + (1.0956 x platelet count [0 when platelets < 1500 x 10^9/L; 1 otherwise]) x 1000).

Three risk groups were identified:

Low-Risk **(score ≤ 780, 40.6% of patients),and**

Intermediate-Risk **(score 781 – 1480, 44.7% of patients) and**

High-Risk **(score ≥ 1481, 14.6% of patients).**

ANNEXURE 3

EUTOS SCORING

The EUTOS score is calculated as

(7 x basophil [%]) + (4 x spleen [cm]).

Two risk groups were identified:

Low-Risk **(score < 87, 79% of patients) and**

High-Risk **(score ≥ 87, 21% of patients)**

The 10EUTOS manuscript also provides the following formula for predicting the probability of no CCgR at 18 months:

exp(−2.1007 + 0.0700 × basophils + 0.0402 × spleen size)/(1 +

exp[−2.1007 + 0.0700 + basophils + 0.0402 × spleen size]).

ANNEXURE 4

CML response criteria

European LeukemiaNet Response Criteria & Response Expectations

Response	Criteria	Frequency of monitoring
Complete Hematologic Response (CHR)	Platelets < 450 x10^9/L, AND White cells < 10 x10^9/L, AND No circulating immature myeloid cells, AND < 5% basophils on differential, AND No palpable splenomegaly	Every 2 weeks until CHR achieved and confirmed, then every 3 months
Minimal Cytogenetic Response (MinimalCyR)	66 - 95% Ph+ cells*	Every 6 months until CCyR achieved and confirmed, then annually
Minor Cytogenetic Response (MinorCyR)	36 - 65% Ph+ cells*	Every 6 months until CCyR achieved & confirmed, then annually
Partial Cytogenetic Response (PCyR)	1 - 35% Ph+ cells*	Every 6 months until CCyR achieved & confirmed, then annually
Complete Cytogenetic Response (CCyR)	No Ph+ cells*	Every 6 months until CCyR achieved & confirmed, then annually
Major Molecular Response (MMR)	BCR-ABL ≤ 0.10% (international scale)	Every 3 months (peripheral blood)
Complete Molecular Response (CMR)	BCR-ABL transcripts non-quantifiable and non-detect-able	Every 3 months (peripheral blood)

* At least 20 metaphases analysed on conventional cytogenetics of bone marrow aspirate

Time (m)	Optimal response	Suboptimal response	Treatment failure
3	CHR plus at least MinorCyR	No cytogenetic response	< CHR
6	At least PCyR	< PCyR	No cytogenetic response
12	At least CCyR	< CCyR	< PCyR
18	At least MMR	< MMR	< CCyR
At any time	Stable or improving MMR	Additional cyto-genetic abnorm-alities in Ph+ cells, loss of MMR, mutation with a low level of insensitivity to imatinib	Loss of CHR, loss of CCyR, mutation with a high level of in-sensitivity to imatinib

ANNEXURE 5

MATERIALS REQUIRED FOR CYTOGENETIC STUDY

1. Marrow Max
2. Colcemid solution (10 μg/mL)
3. Hypotonic solution: KCL (0.56%), distilled H_2O (Mix 8:2)
4. Fix: Methanol, Glacial Acetic Acid (Mix 3:1)
5. Centrifuge tubes, 15 mL
6. T-25 flasks
7. Sterile transfer pipettes
8. Nonsterile transfer pipettes
9. Eppendorf pipette
10. Incubator, 37ºC
11. Centrifuge
12. Microscope slides
13. Oven, 90ºC
14. Phase microscope (optional)
15. Brightfield microscope

MASTER CHART

S.NO.	AGE	SEX	SYMP	Tc d1	Tc 3	Hb- D1	Plt	R/L	SS	Pp	PT	APTT	BMA	Kar	Gene	Course
1	45	F	f	484000	4700	10	735000	N	15	21	14	40	CML CP	Ph	210	g
2	44	M	s.n	300000	7000	9.3	408000	N	20	21.5	21.9	74.3	CML CP	Ph	210	g
3	45	M	fati	218400	7000	9.9	84000	N	17	18	14	37.8	CML CP	M ACA 1	210	p
4	39	M	f,p	240000	5600	13.4	220000	N	20	19	13.4	36.1	CML CP	Ph	210	g
5	43	M	fati	292700	7500	11.7	274000	N	19	20	13.4	38.4	CML CP	Ph	210	g
6	65	F	p	161000	6500	7.9	418000	N	20	21	15	52.3	CML CP	Ph	210	g
7	49	M	f	256000	5400	7.1	1,002,000	N	22	19	14.9	61.7	CML CP	Ph	210	g
8	45	F	f,p	248600	5200	8.3	470000	N	20	23	12.1	33.9	CML CP	Ph	210	g
9	46	M	f, LN	253600	5500	8	217000	N	22	25	12.6	37.2	CML CP	M ACA 2	210	p
10	24	F	p	229000	9200	8.3	1,065,000	N	18	21	12.6	34.3	CML CP	Ph	210	g
11	24	M	f, p	202000	5500	11.4	706000	N	19	21	13	37	CML CP	Ph	210	g
12	36	M	p	220000	7000	9.1	293000	N	21	19	12	43	CML CP	Ph	210	g
13	49	M	f, p	78900	56800	9.7	125000	N	>20	21	13	40.3	CML CP	M ACA 3	210	g
14	26	M	fati	146700	4800	9.7	347000	N	17	21	12.1	41.6	CML CP	Ph	210	g
15	52	M	f	208700	14300	9.2	319000	N	19	21	12.5	42	CML CP	Ph	210	g
16	60	M	p	142100	5200	9.8	374000	N	14.5	20	13.1	39	CML CP	Ph	210	g
17	60	F	f	97600	3200	9	183000	N	13.5	19	12.5	40	CML CP	Ph	210	g
18	45	M	fati	152900	5100	9.5	661000	N	19	18	16.7	36.5	CML CP	v,t	210	g
19	35	M	f	240000	5000	10.9	364000	N	18	30	14.9	44.8	CML CP	Ph	210	g
20	24	M	p	185600	5100	9.8	171000	N	14	20	16.2	43.4	CML CP	Ph	210	g
21	41	M	f	242000	9100	8.5	130000	N	22	17	13	45	CML CP	Ph	210	g
22	24	M	p	176000	7300	10.6	203000	N	17	19	14	39	CML CP	Ph	210	g
23	24	M	p	288000	5600	10.2	223000	N	>20	21	24	36.3	CML CP	del Y	210	g
24	57	F	p, f	208300	5800	10.5	360000	N	19	18	12.5	37.3	CML CP	Ph	210	g
25	46	F	fati	298700	3700	8.5	345000	N	19	19	11.8	26.4	CML CP	Ph	210	g
26	43	M	p	147700	3900	8.9	408000	N	19	14	16.7	36.5	CML CP	Ph	210	g
27	30	M	p	164300	4200	11.2	378000	N	18	35	12.2	32.3	CML CP	Ph	210	g
28	29	M	fati	160000	119100	9.2	180000	N	20	20	13	33.8	CML CP	ph	210	g
29	40	F	f	185000	6000	10.3	671000	N	21	21	14.2	45	CML CP	M ACA 4	210	p
30	29	F	p	180700	5700	8	31800	N	19	12	12.2	36.7	CML CP	Ph	210	g

KEY TO MASTER CHART

Symp	–	Presenting Symptoms
Tc d1	–	Total WBC Count Day 1
Tc 3 mon	–	Total WBC Count 3 months
Hb	–	Haemoglobin
Plt	–	Platelet Count
SS	–	Spleen Size
Pp	–	Portal pressure
PT	–	Prothrombin Time
APTT	–	Activated Partial Thromboplastin Time
BMA	–	Bonemarrow Aspiration
F	–	Fever
P	–	Abdominal Pain
Fati	–	Fatigue
s.n	–	Subcutaneous Nodules
LN	–	Lymphadenopathy
Ph	–	Standard t(9,22)
M ACA 1	–	46xy,t(9,22)(q34;q11.2)[3]/48,idem,+8,inv(12),+21[22]
M ACA 2	–	46xy,t(9,22)[8]/49xy,idem,+8,+19,t(9,22)[18]
M ACA 3	–	46xy,t(9,22)[14]/ 47, idem, +der(22) t(9,22)[22]
M ACA 4	–	46xx,-1,der(6)t(1,6) t(6,13), der(7)t(1,7), t(9,22), del(10) +mar[23] /46XX[2]
V,t	–	46xy,t(3,6,9,22)(p13;p21;q34;q11.2)[25]
delY	–	46xy,t(9,22)(q34;q11.2)[3]/45x, idem, -y[22]
210	–	210 KD Protein
Gene	–	Bcr Abl Quality
Kar	–	Karyotype
P	–	Progression of Course
G	–	No Progression of Course
Acc	–	Accelaraion Phase
R/L	-	Renal / Liver Function Test